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SECTION MEETINGS

CLEVELAND	
Academy of Medicine	October 12, 1951
PACIFIC COAST	
University of California	October 10, 1951
SOUTHERN CALIFORNIA	
California Institute of Technology	October 11, 1951

Studies in Non-Protein Nitrogen. I. A Convenient Method for Measuring Urea in Blood. (19073)

Raymond H. Owings and Emanuel E. Mandel. (Introduced by P. K. Bondy.)
(With the technical assistance of Miss Florence Jones.)

From the Communicable Disease Center, Public Health Service, Federal Security Agency, Atlanta, Ga.

The acid-heat hydrolysis method of measuring urea in biological fluids, initiated by Benedict and Gephart(1) and further developed by later workers(2-4), has recently been revised by Kibrick and Skupp(5). The procedure as applied by these authors to serum and plasma filtrates would appear to represent a highly convenient way of determining urea nitrogen. They did not extend the method to whole blood filtrates because of cloudiness which developed after addition of Nessler's solution, an observation known since the work of Folin and Denis(6). However, for reasons of convenience and ease of manipulation, the routine use of whole blood often is preferable in the clinical laboratory. Indeed, application of the method to whole blood was thought to be possible by employing the combined gluconate-persulfate nesslerizing reagent which was shown by Gentzkow(7) to prevent turbidity in filtrates from blood samples subjected to urease hydrolysis. This expectation was confirmed in our experiments.

Experimental. In order to test the accuracy of Kibrick and Skupp's method, it was applied at first to prepared urea nitrogen solutions. One ml of urea solution (or of distilled water for the blank) and 0.5 ml of 1 M phosphoric acid in a tin foil-covered test tube were subjected to either 15, 20 or 22 lb of pressure for

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1. Benedict, S. R., and Gephart, *J. Am. Chem. Soc.*, 1908, v30, 1760.

2. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, v38, 81.

3. Clark, E. P., and Collip, J. B., *ibid.*, 1926, v67, 621.

4. Leiboff, S. L., and Kahn, B. S., *ibid.*, 1929, v83, 347.

5. Kibrick, A. C., and Skupp, S., Proc. Soc. Exp. Biol. and Med., 1950, v73, 432.

6. Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, v26, 473.

7. Gentzkow, C. J., *ibid.*, 1942, v143, 531.

TABLE I. Percent Recoveries of Urea Nitrogen from Prepared Urea Solutions.

Urea N solutions	Kibrick-Skupp method			New method			
	15	20	22	15	20	22	Lb pressure
	90	60	90	90	60	90	Min
mg/100 ml	% recovered						
11.66	94.1	97.4	102.2	90.6	89.5	100.3	
23.32	93.2	93.7	100.8	86.7	87.9	98.6	
46.64	93.3	93.5	97.7	85.8	88.5	99	
75	92	92.1	—	84.1	87.3	98.7	

Each % value represents average of triplicate analyses. The overall standard experimental error was computed as: Overall S. E. = $\sqrt{\frac{\Sigma_r \Sigma_n (x-m)^2}{r(n-1)}}$, where x represents the individual value; n, the number of replicates per analysis; m, the arithmetical mean of replicates, or $\frac{\Sigma x}{n}$; r, the number of sets of replicates. Overall S. E. for Table I was 1.12.

TABLE II. Percent Recoveries of Urea Added to Serum.

Urea N added to serum containing 11 mg/100 ml	Kibrick-Skupp method			New method			
	15	20	22	15	20	22	Lb pressure
	90	60	90	90	60	90	Min
mg/100 ml	% recovered						
30	91.6	93.3	98.9	91.6	99.7	101	
50	89.8	91.9	97.3	89.2	93.6	97	
100	87.7	92.6	97.8	83.1	95.6	97.8	

In setting up recovery experiments, urea standard solutions of appropriate concentrations were substituted for some of the distilled water used in making tungstic acid filtrates. Each figure expressing % recovery represents an average of the analyses of three filtrate aliquots. Overall S. E. = 1.19.

60 to 90 minutes (Table I) in a hospital autoclave.* After cooling, 1 ml of 1 N sodium hydroxide was added and thereafter distilled water to the 10 ml mark on the tube. This was followed by 1 ml of Nessler's reagent prepared according to Koch and McMeekin (8). After allowing the well-mixed solution to stand for 10 minutes, readings were taken within 10 minutes in a Beckman model DU spectrophotometer at a wave length of 500 millimicra and in a Klett-Summerson colorimeter with filter 54. (In view of the greater precision of the former instrument, only results obtained by it will be presented.) Standard solutions containing 2 and 5 mg of nitrogen, as ammonium sulfate, per 100 ml, treated in like manner but without the autoclaving step, served as the bases for photometric comparison and calculation of results.

Results. It is apparent from Table I that under the conditions of pressure and time recommended by Kibrick and Skupp(5), namely 15 lb and 90 minutes or 20 lb and 60 minutes, recoveries of urea nitrogen were quite deficient. However, they were very satisfactory with the use of 22 lb for 90 minutes. This was true with and without the modification using the gluconate-persulfate reagent described below. Data on the recov-

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* A horizontal, direct steam-heated type of 14000 cu in. capacity made by the American Sterilizer Co., Erie, Pa. (Company names are used as a means of identifying the products under discussion, and do not represent an endorsement of the products by the Public Health Service.)

8. Hawk, P. B., Oser, B. L., and Summerson, W. H., Practical Physiological Chemistry, 12th Edition, The Blakiston Co., Philadelphia and Toronto, 1942, p. 1230.

TABLE III. Percent Recoveries of Urea Added to Whole Oxalated Blood.

Urea N added to blood, mg/100 ml	Urea N found, mg/100 ml	% urea N recovered	Hydrolyzing conditions
0	7.5	—	15 lb
27	31.1	87.4	90 min
40.5	37.6	74.4	
81.1	51.3	54	
0	15.6	—	20 lb
19.9	35.8	101.5	90 min
39.9	54.9	98.7	
59.8	75.9	100.7	
99.7	114.9	99.6	
0	12.7	—	22 lb
30	43.8	103.5	90 min
50	62.5	99.6	
100	111.8	99.1	
150	167	102.8	

See footnote of Table II. Overall S. E. = .95.

ery of urea added to serum or blood as listed in Tables II and III, respectively, warrant the same conclusions. At 20 lb or more applied for 90 minutes, recoveries were practically complete.

These results appeared to indicate that hydrolysis of urea may not be complete at the pressure values originally suggested(5); hence they were increased in the procedure as finally adopted.

New method. One ml of tungstic acid filtrate from either whole blood, plasma or serum and 0.5 ml of 1 M phosphoric acid in a tube covered with tin foil are placed in an autoclave or pressure cooker and exposed to a minimum of 20 lb of pressure for 90 minutes. Into the cooled tube, 1 ml of 0.9 N sodium hydroxide is pipetted,† followed by distilled water to the 10 ml mark. After mixing, 2 ml of Gentzkow's nesslerizing reagent(7) are added (2 parts of Folin Nessler's solution(2), 1 part of 2.5% aqueous nitrogen-free potassium persulfate and 1 part of 1% aqueous potassium gluconate. The mixture must be used within 15 minutes. Both the persulfate and gluconate solutions must be made up fresh weekly and kept under refrigeration). The sample is shaken, allowed to stand for 15 minutes and read within the next 90 minutes in a spectrophotometer or photoelectric colorimeter at a wave length of between 490 and 510 millimicra. A distilled water blank and an ammonium sulfate standard (containing 2 mg of nitrogen per 100 ml) are treated identically except that the standard is not autoclaved. For convenient computation of results, a calibration curve may be prepared by means of a series of ammonium sulfate solutions ranging in concentration between 1.5 and 8 mg of nitrogen per 100 ml. Within this range there exists a straight-line relationship between concentration and optical density. Filtrates of blood samples containing more than 80 mg of urea nitrogen per 100 ml must be diluted with distilled water and the results multiplied by the dilution factor. In order to test for completeness of hydrolysis, particularly when the pressure gauge is not reliable, it is desirable to include a urea solution of

TABLE IV. Comparison of Blood Urea Nitrogen Values as Determined by the New and by the Urease Methods.

	mg of urea N per 100 ml		
	Urease methods		
Blood sample No.	Gentzkow's(7)	Karr's(9)	New method
1	5	3	6.5
2	9.3	8	10.3
3	9.9	7	9.7
4	10.7		10.9
5	13.4		13.8
6	14.8	13	17.3
7	15.5	12	14.5
8	16.2	18	20.8
9	17.4	13	15.9
10	22.8	20	22.8
11	37.3	33	32.5
12	38.8		39
13	40		39.7
14	53.3		55.5
15	53.5		54.5
16	59	59	59.8
17	61.8		62
18	75	70	77.6
19	75		73.4
20	99.6	110	104

Values given for Gentzkow's and for the new method are averages of duplicate analyses determined by means of the Coleman Jr. Spectrophotometer. Overall S. E. for the former method was estimated to be .22, for the latter, .16. Values for Karr's method represent single analyses performed in the course of routine hospital laboratory work with the use of a Klett-Summerson colorimeter.

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† This results in a pH of about 7.0 while the use of 1 N sodium hydroxide, as previously recommended by Kibrick and Skupp(5), produces an unduly high pH which may enhance turbidity after nesslerization.

9. *Ibid.*, p. 505.

known concentration in the series of unknowns analyzed.

Application of the method to a series of blood samples and comparison with Gentzkow's(7) and Karr's(9) methods using urease hydrolysis (Table IV) resulted in satisfactory agreement of values.

Summary. The acid hydrolysis method of measuring urea has been revised so that it will furnish accurate and reliable results when applied to either whole or cell-free blood.

The computation of standard errors was carried out by Mr. Myron J. Willis of the Statistics Section, Epidemiologic Services, Communicable Disease Center.

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Abrupt Improvement of Serum Electrophoretic Pattern in Nephrosis After ACTH-Induced Diuresis. (19074)

SIDNEY E. LENKE AND HENRY M. BERGER. (Introduced by Albert E. Sobel.)
From the Mattie Billings Lowe Laboratory, Jewish Hospital of Brooklyn, N. Y.

This paper deals with the partial correction of the abnormal electrophoretic pattern, which is characteristic of the serum in nephrosis(1-4), in patients who responded to ACTH with intense diuresis. In the nephrotic syndrome there occurs a progressive accumulation of fluid in the interstitial spaces and the serous cavities, the edema increasing until, quite unpredictably, it is relieved by massive diuresis. Until recently the onset of diuresis has been fortuitous, and it has therefore been difficult to follow the chemical and physical changes which occur in blood just before and after diuresis. It is now possible to produce diuresis in many nephrotic patients with a variety of drugs such as ACTH, cortisone, urea, cation exchange resins, and occasionally by nitrogen mustard. The effect of the diuresis thus produced upon some chemical constituents of the blood has been described(5-7). This paper describes the marked changes observed in the electrophoretic pattern of the serum proteins.

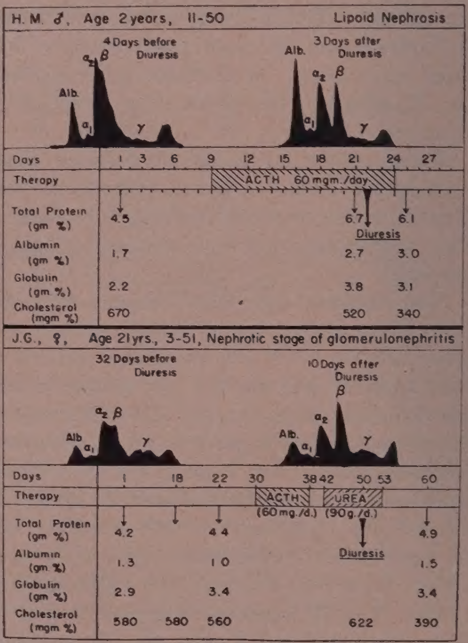

FIG. 1. Total protein, albumin, globulin results listed were determined by chemical analyses.

Methods and materials. Observations are presented from 4 cases, 3 infants suffering from lipoid nephrosis and one adult female, 21 years of age, suffering from chronic glomerulonephritis. Her nephrotic syndrome

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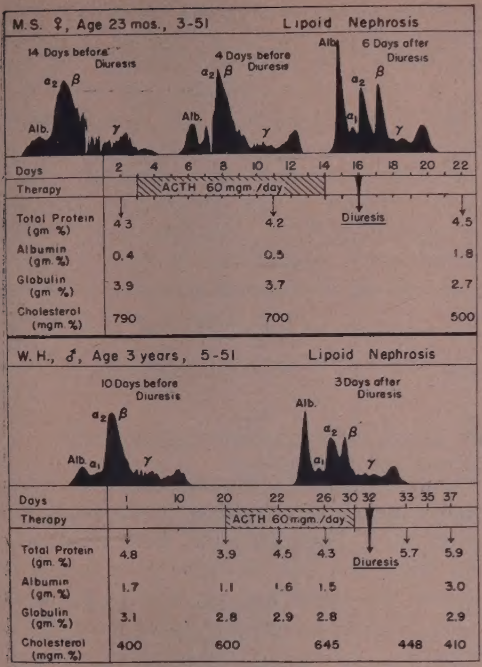
5. Block, W. M., Jackson, R. L., Stears, M. P., *Ped.*, 1948, v7, 33.
6. Schwartz, H., Kohn, J. L., *Am. J. Dis. Child.*, 1938, v49, 579.
7. Gotfried, S. P., Steimen, J. T., and Kramer, P., *Am. J. Dis. Child.*, 1946, v74, 283.



Total Protein (gm %)	4.3	4.2	Diuresis	4.5
Albumin (gm. %)	0.4	0.5		1.8
Globulin (gm %)	3.9	3.7		2.7
Cholesterol (mgm. %)	790	700		500

Total Protein (gm. %)	4.8	3.9	4.5	4.3	Diuresis	5.7	5.9
Albumin (gm. %)	1.7	1.1	1.6	1.5			3.0
Globulin (gm. %)	3.1	2.8	2.9	2.8			2.9
Cholesterol (mgm. %)	400	600		645		448	410

FIG. 2. Total protein, albumin, globulin results listed for W.H. were determined chemically. Those listed for M.S. were determined by electrophoretic calculations.

began late in pregnancy, 6 months before the present observations. ACTH alone was used to produce diuresis in the 3 cases of lipoid nephrosis (W.H., H.M., M.S.). Sixty mg of ACTH was given intramuscularly in divided doses each day for 8 to 15 days (as indicated in Fig. 1 and 2).

In the adult case (J.G.) when ACTH failed to produce diuresis in 8 days, 4 additional days were allowed to elapse and then urea was administered orally (90 g daily). Diuresis occurred during the administration of urea.

The following analyses were done before therapy and after diuresis: 1. Electrophoretic analysis of the serum proteins; 2. Total proteins (by the micro-Kjeldahl method); 3. A/G ration (electrophoretically calculated); 4. Total serum cholesterols. The technic used in the electrophoretic analysis has been described(8,9). All analyses were made using a barbiturate buffer of pH 8.6, ionic strength 0.1. The apparatus used was the Tiselius electrophoresis apparatus.

Results. It is evident from Fig. 1 and 2 that within a few days after diuresis a remarkable change occurred in the serum proteins of these 4 cases. This change as determined electrophoretically was toward the normal.

The most notable change occurred in the region of the α_2 and β globulins. This is the region which characteristically fails to show resolution in the nephrotic serum before diuresis, appearing as an amorphous, roughly outlined area. After diuresis this area showed excellent resolution of these α_2 and β globulins. The total of α_2 and β globulins decreased after diuresis, but still remained above normal limits (Table I).

Concurrently there was a rise in the serum albumin, varying from 16.7% to 350%. The concentration of the protein components is presented in Table I. Calculations of the α_2 and β globulins has not been attempted in the patterns before diuresis because of the characteristic lack of resolution of these peaks. The α_1 and γ globulins seem to show no significant change after diuresis.

The total protein showed a moderate increase after diuresis, an increase reflecting the additional albumin, as well as in one case a rise in the total globulin. In 3 cases the total globulin decreased after diuresis.

The serum cholesterol levels showed a marked decrease in all 4 cases following diuresis (Fig. 1 and 2), and the gross lipemia which is typical of nephrotic serum became visibly clearer after diuresis. However, even after diuresis some lipemia is evident and the serum cholesterol values, even though markedly decreased, remain considerably above normal limits.

Discussion. The marked change in the electrophoretic serum protein pattern which occurred in these nephrotic patients after diuresis, the rise in the albumin, the decrease in the α_2 and β globulins, with clearer resolution of these globulins, is surprisingly abrupt. In this laboratory's experience with over 700

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8. Weiner, A. S., Berger, H. M., and Lenke, S. E., *Lab. Digest,* 1951, v14, 11.

9. Lenke, S. E., and Berger, H. M., *Lab. Digest,* 1951, v15, 4.

TABLE I. Concentration of Protein Components in Serum Before and After Diuresis as Determined Electrophoretically.

Case	Date	T.P.	Alb.	Glob.	α_1	α_2	β	γ
S.	3/21	4.3	.4	3.9				
S.	3/30	4.2	.5	3.7				
Diuresis								
S.	4/11	4.5	1.8	2.7	.3	1.1	1.1	.2
H.	4/28	4.5	.5	4	.2	——3.5——		.3
Diuresis								
H.	5/10	5.9	2.1	3.8	.3	1.6	1.3	.6
G.	4/12	4.1	.6	3.5	.3	——2.4——		.9
Diuresis								
G.	5/18	4.4	.7	3.7	.3	1.2	1.6	.6
M.	11/3	5.5	1.1	4.4	.2	——4.2——		
Diuresis								
M.	11/28	5.5	1.8	3.7	.3	1.7	1.3	.5

electrophoretic patterns in a variety of clinical investigations, we have been impressed with the essential stability of an individual's serum protein pattern. The hypoalbuminemia of nephrosis has been singularly refractory to improvement, even after the daily injection of large amounts of albumin. The improvement after ACTH-induced diuresis is therefore notable, since no such change occurs even with diuresis induced by giving 50 g of salt-free human albumin intravenously for 15-30 days* (4,10). Work is in progress to ascertain whether the increased serum albumin is due partly or wholly to a decreased proteinuria.

One possible source of the sudden increase in the serum albumin is the interstitial fluid. Though relatively protein-poor, a diuresis of 5 liters should yield at least 25 g of albumin to the body. Another possibility, that some of the increased albumin which was noted after diuresis was actually present in the serum before diuresis, but migrated in the region of the α_2 or β globulins due to an abnormal electrostatic surface charge, is now under investigation in this laboratory.

The second impressive change after diuresis is the decrease of the α_2 and β globulins and the marked improvement in the resolution of these peaks. This may be considered as additional evidence of the importance of lipids and protein-lipid complexes in the nephrotic syndrome. These globulins have an affinity for lipids and seem to serve as a transport system for lipids and fat soluble pigments (11) much as hemoglobin serves to transport oxygen.

The improvement in the α_2 and β globulin region of the electrophoretic pattern occurs as the serum cholesterol and gross lipemia improve. It may be assumed that the physiological mechanism which produces the salutory diuresis, produces as well, an improvement in the basic lipid disturbance of nephrosis. That this occurs without ACTH or cortisone has not been observed by us, nor reported by those who used nitrogen mustard or urea alone.

A notable difference is evident between the response of infants suffering from lipoid nephrosis and the adult case of glomerulonephritis. Although the resolution of the globulin peaks improved in both, the adult case showed only a slight rise in the serum albumin. Experiments are now being pursued to ascertain whether this distinction is repeatable and whether it is due to the nature of the two diseases or to a specific effect of ACTH on the serum albumin. (The diuresis in the adult case occurred some time after ACTH, during the administration of urea).

Conclusion. 1. A striking change is noted in the electrophoretic pattern of the serum in nephrotic patients soon after diuresis induced by ACTH, or ACTH plus urea. The pattern which is characteristic of nephrotic serum (low albumin and large, unresolved α_2 and β globulin peaks) reverts toward the normal after diuresis. The serum albumin rises sharply and the α_2 and β globulin region shows clear resolution. 2. Possible explanations of these abrupt changes in the electrophoretic patterns and concentrations of serum proteins are discussed.

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* Albumin excretion was followed in a later group of patients and will be presented in another paper.

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11. Blix, G., *J. Biol. Chem.*, 1942, v137, 485.

We are grateful to Dr. Dan Moore of the College of Physicians and Surgeons, Columbia University, Dr. William Dock, Professor of Medicine, State University Medical Center at New York, Dr. Albert E. Sobel, Head of the Department of Biochemistry and Dr. Benjamin Kramer, Chief of Pediatrics, at the Jewish Hospital of Brooklyn, for their help in preparing this report.

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Acetoacetate-Induced Changes in Blood Lactic and Blood Ascorbic Acids; Prevention by Insulin and Amellin. (19075)

Madhab Chandra Nath and Chitta Haran Chakrabarti.

From the University Department of Biochemistry, Nagpur, India.

Diabetes is frequently associated with acidosis, a symptom characterized by a lowering of the blood alkali reserve. It has recently been shown by the authors(1) that the injection of β-hydroxybutyrate into normal rats caused a depletion in liver and muscle glycogen and an increase in the tissue lactic acid. Green and Brosteaux(2) reported that pyruvic acid almost completely inhibits the enzymatic oxidation of lactic acid *in vitro,* and Holmes and Holmes(3) have observed a direct relationship between the blood sugar value and the brain lactic acid content in both hypo and hyperglycemic conditions. The increase of lactic acid in diabetes has been observed by Maranon and his associates(4) and Genes(5). Marenzi(6) has observed that the injection of anterior pituitary extract into dogs causes an increase in blood sugar and blood lactate values. Vit. C has been found by Sigal and King(7) and Banerjee and Ghosh(8,9) to play a role in carbohydrate metabolism. Sigal and King(7) have shown that the successive stages of Vit. C depletion are associated with a corresponding rise in the fasting blood sugar level and a distinctly lowered glucose tolerance; the administration of Vit. C restores the carbohydrate metabolism to normal. The injection of acetoacetate in a dose of 18 mg per 100 g body weight has recently been reported by Nath and his associates(10) to cause a marked depression in the blood Vit. C level of rabbits. An attempt was therefore made to study the cumulative effects of acetoacetate on the lactic acid and the Vit. C contents of the blood of rabbits.

Experimental. In the first set of experiments (Group A), 6 healthy male rabbits

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TABLE I. Effect of Acetoacetate on Blood Lactic Acid and Ascorbic Acid.

Group A							Group B			
			Lactic acid, mg/100 cc blood		Ascorbic acid, mg/100 cc plasma				Lactic acid, mg/100 cc blood	
No. of rabbits	Day of acetoacetate inj.*	Days of treatment with amellin	Avg value	Stand. dev.	Avg value	Stand. dev.	No. of rabbits	Day of acetoacetate inj.*	Avg value	Stand. dev.
6	0		6.2	.86	2.5	.36	20	0	7.9	1.01
6	10		8.9	2.16	2.2	.20	20	10	11.3	1.54
6	20		12.3	2.74	1.7	.10	19	30	18.5	1.48
6	40		15	2.80	1	.06	18	50	24.7	1.94
6	60		16	2.28	.83	.14	16	70	30.1	1.57
4	80	20 (10 mg daily)	7.2	.88	—	—	15	90	36.7	1.76

* Daily dose of acetoacetate injected: Group A. 1st to 10th day—25 mg/kg; 11th to 20th day—50; 21st to 30th—75; 31st to 40th—100; 41st to 50th—125; 51st to 60th—150; 61st to 80th—175.

Group B. 1st to 10th—50 kg; 11th to 30th—100;31st to 50th—150; 51st to 70th—225; 71st to 90th—250; 91st to 100th—275; 111th to 120th—300.

(each weighing about 2 kg) with a fasting blood sugar level between 108-112 mg/100 cc were injected daily with acetoacetate (intraperitoneally) in the doses given in Table I.

In a second group of 20 animals (Group B) higher doses of acetoacetate were injected for a considerably longer period. The animals were anaesthetized with Nembutal, the blood was collected periodically and lactic acid was determined by the method of Barker and Summerson(11). Preliminary determinations on the plasma Vit. C were carried out in Group A by the method of Bessey(12).

Liver glycogen was shown previously(13) to be markedly depleted by injection of acetoacetate, but restored to a considerable extent by the simultaneous injection of acetoacetate plus either insulin or amellin.* Since blood lactate may be utilized to form liver glycogen, a preliminary study was carried out (Group A, Table I) to determine the effect of amellin on the blood lactate level. A more detailed study was carried out in Group B. After these animals had received acetoacetate injections for 90 days, the rabbits were divided into 3 groups; the first was kept as the control, the second given 10 mg of amellin per kg/day and the third 0.5 unit of insulin per kg/day. The acetoacetate injections were continued and the lactic acid content of the blood was determined after an additional 20 and 40 days. The results are shown in Table II.

Discussion. The results suggest that acetoacetic acid may inhibit the oxidation of lactic acid. However, the large increase in blood lactic acid could have been the result of non-utilization or of partial utilization of lactic acid by the liver for the synthesis of glycogen. This deficiency may be due to inactivation of endogeneous insulin, induced by the injection of ketone bodies(14). Severe depletion of liver glycogen has recently been observed by Bondy and Sheldon(15) in human diabetics by liver biopsy. Foglia(16) has shown that in subtotally depancreatectomized rats, the liver could not form glycogen during severe diabetes. That insulin lack may be associated with increased blood lactic acid is indicated by the work of Petiteau(17), who observed that the blood lactate increased much faster after hepatectomy in pancreatectomized dogs than in dogs with an intact

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11. Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, v138, 535.

12. Bessey, O. A., *J. Biol. Chem.*, 1938, v126, 771.

* This is a complex protein-like plant substance which contains a purine base, a thio sugar, and a phosphate group(13).

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16. Foglia, V. G., *Rev. Soc. Argentina Biol.*, 1945, v21, 7.

17. Petiteau, C., *Compt. rend. Soc. Biol.*, 1938, v127, 1105.

TABLE II. Effect of Insulin and Amellin in Utilizing Blood Lactic Acid.

Group No.	No. of animals	Days of aceto-acetate inj.*	Days of treatment with— Amellin (10 mg/kg)	Days of treatment with— Insulin (.5 U/kg)	Lactic acid, mg/100 cc blood Avg values	Lactic acid, mg/100 cc blood Stand. dev.
I	5	90			36.2	2.04
,,	5	110			54.2	3.31
,,	3	130			62.4	1.64
II	5	90	0		37.1	1.63
,,	5	110	20		22.5	1.50
,,	5	130	40		7.6	.46
III	5	90		0	36.9	1.43
,,	5	110		20	11.8	.91
,,	5	130		40	29.6	.87

* Dose as in Table I (Group B).

pancreas.

Banerjee and Ghosh(8) observed that guinea pigs placed on a scorbutic diet for 21 days showed a decreased glucose tolerance as compared with controls receiving a diet supplemented with ascorbic acid. Banerjee (9) observed that in scorbutic animals the insulin content of the pancreas was reduced and the β-cells showed definite signs of degeneration. Mosenthal and Loughlin(18) found plasma Vit. C levels of less than 0.8 mg % in 43% of more than 100 diabetic children studied. We have observed the diminution of ascorbic acid in the blood of our experimental animals, even though they received sufficient Vit. C along with their food.

After 90 days of administration of sodium acetoacetate, the blood lactate was markedly increased. Injection of amellin for 40 days, even along with the acetoacetate, reduced the lactic acid to normal. Insulin produced a transient beneficial effect in the utilization of lactate during the first 20 days, but was unable to prevent the accumulation of blood lactate when the administration of acetoacetate plus insulin was prolonged (40 days).

Summary. 1. The daily injection of 50 mg/kg of sodium acetoacetate into normal rabbits in increasing doses markedly increased the blood lactate level to an average of 36.7 mg/100 cc (control = 7.9 mg/100 cc) after 90 days. 2. In a second group of animals receiving about half the dosage, the plasma ascorbic acid decreased from 2.50 to 0.83 mg/100 cc after 60 days. 3. Simultaneous injection of amellin prevented the accumulation of blood lactate. In animals which had received sodium acetoacetate for 90 days, amellin restored the blood lactate level to normal within 40 days, in spite of the continued injection of increasing amounts of the acetoacetate. 4. In an experiment otherwise identical, insulin was not as effective as amellin; when it was injected, the blood lactate diminished initially but later increased. 5. The amellin treated animals were healthier than the insulin treated ones.

The authors wish to thank Dr. Arnold Lazarow of Western Reserve University for his keen interest in their work.

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Agar Layer Method for Production of High Titer Phage Stocks.* (19076)

M. Swanstrom and M. H. Adams. (Introduced by Colin M. MacLeod.)

From the Department of Microbiology, New York University College of Medicine

Bacteriophage stocks prepared by the lysis of fluid bacterial cultures usually average 10^{10} infectious particles per ml and rarely exceeded 10^{11}/ml. A method of achieving higher virus concentrations than this is desirable. Luria(1) suggested a modification of a method originally used by Hershey(2) which under proper conditions gives a virus stock about 10 times as concentrated as those obtainable in fluid cultures. This method has been briefly described(3) and has been used successfully by Cohen(4) and by Marshak(5) for the preparation of purified phage suspensions. The purpose of the present paper is to describe the method in general terms and to point out those significant variables which should be investigated and controlled by anyone wishing to use the method with a new phage-host cell system.

Materials and methods. In essence the method consists in the lysis of a dense suspension of the host bacterium by the phage in a thin agar layer which has been poured over a much thicker base layer of nutrient agar. When bacterial lysis is complete the upper agar layer is scraped from the plates and the phage is extracted with minimum amounts of broth. A sample experiment using coli phage T2r+ will illustrate the method. The basal agar contains 0.8% Difco nutrient broth, 0.5% NaCl and 1.5% agar. About 40 ml of melted agar is poured into each petri dish and allowed to harden with the dishes resting on a leveled sheet of plate glass. The soft agar for the agar layer differs only in the agar content which is 0.7%. The melted soft agar is distributed in 2.5 ml amounts in test tubes and cooled to 46°C. The bacterial inoculum is prepared by washing the overnight growth from an agar slant with 2 ml of broth giving a concentration of about 5×10^9 bacteria/ml. Each tube of agar is inoculated with 5×10^8 bacteria and 7×10^5 T2r+ phage particles, and then is poured over the base agar in a petri dish resting on leveled plate glass. After the agar layer has hardened the plates are incubated at 37°C overnight, and on examination the bacteria are found to be completely lysed. Five ml of broth are added to each plate and the agar layer is scraped off with a sterile glass rod into a beaker. The broth and agar from 10 plates are pooled, transferred by pipette to centrifuge tubes and shaken vigorously for a few minutes to break up the agar and extract the phage. The agar is sedimented in a horizontal centrifuge at 2000 RPM and the slightly opalescent supernatant fluid decanted and assayed for virus content. The yield in this experiment was 36 ml assaying 10^{11} infectious particles per ml. For comparison Hook *et al.*(6) prepared T2r+ phage on a large scale in fluid media, obtaining from 5×10^9 to 2×10^{10} infectious particles/ml. The yield per ml of medium used is about the same in the two methods but the concentration achieved is from 5 to 20 times greater in the agar layer method.

Typical yields obtained by this method for the various coli phages of the T series are given in Table I, together with the phage inoculum used per plate. In each case about 40 ml of virus stock were recovered from the

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* Aided by a grant from the National Foundation for Infantile Paralysis. This paper is the essence of a thesis submitted by M. Swanstrom in partial fulfillment of the requirements for the degree of M.S. in the Graduate School of Arts and Sciences of New York University.

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3. Adams, M. H., Methods in Medical Research, 1950, v2, 12, Chicago, Year Book Publishers.

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TABLE I. Yields of Phage Obtained by Extraction of Soft Agar Layers of Lysed Plates. Pooled agar layers from 10 plates were shaken with 50 ml of broth and centrifuged to remove agar. The average recovery was 40 ml.

Phage used	Inoculum per plate	Phage assay per ml	Yield per plate
T1	4×10^3	7×10^{11}	2.8×10^{12}
T2r$^+$	7×10^5	1.1×10^{11}	4.5×10^{11}
T3	2×10^3	1.5×10^{11}	6×10^{11}
T4r	2×10^5	1.4×10^{11}	5×10^{11}
T5	9×10^5	1×10^{12}	4×10^{12}
T6r$^+$	4×10^5	4.7×10^{11}	1.8×10^{12}
T7	4×10^3	1×10^{11}	4×10^{11}

pooled extracts of 10 plates. The yields with phage T1 and T5 have been consistently greater than with other viruses. The T4r strain used in these experiments was a variant of T4 obtained from A. H. Doermann. In earlier experiments yields with this strain were very poor being of the order of 3 x 10^{10}/ml, but even this was much better than the yields in broth lysates, about 4 x 10^9/ml. Therefore, this strain of T4 phage was used in a systematic study of those variables which might affect the concentration and yield of phage obtainable by this method. It should be noted that different strains of the same phage type may differ considerably in the yields obtained.

Experimental. Since a limited number of virus particles are liberated from each infected bacterium (burst size), it is evident that the yield obtainable from each inoculated petri dish is dependent on the number of bacteria which can be produced before all are lysed by the virus, while the concentration of virus obtainable is dependent on the volume in which the bacterial population can be induced to grow. The nutritional state of the host bacterium is another factor affecting the yield of virus. Bacterial overpopulation results in reduced virus yield per infected cell. Bacterial starvation ultimately results in failure of lysis which is the principal reason why single plaques do not increase indefinitely in size. Another factor limiting the virus yield is the readsorption of excessive amounts of virus to unlysed bacteria. All such particles adsorbed above the one needed to infect the bacterium are lost and contribute nothing to the final yield(7). This loss by readsorption increases in proportion to both bacterial and virus concentrations. From these observations one might anticipate that the optimal phage inoculum would consist of barely enough virus particles to give confluent plaques when the plate has been incubated long enough to give maximum plaque size. This would permit maximum growth of the bacterial population before all were overwhelmed by the virus. With virus T4r confluent lysis is barely attained with 2 x 10^4 particles per plate. The effect of varying phage inocula on the phage yield was determined while keeping all other experimental conditions constant. Over the range of inocula from 3 x 10^4 to 3 x 10^5 phage particles per plate the phage yield remained constant within a factor of 2 averaging 4 x 10^{10} particles per plate. With inocula below 2 x 10^4 per plate bacterial lysis was incomplete and the phage yield was markedly decreased. With inocula above 6 x 10^5 per plate the phage yield decreased presumably because all bacteria were lysed before the optimum bacterial population was reached. Needless to say, the optimal phage inoculum is different with different phage strains, being only 4 x 10^3 per plate for a large plaque phage such as T1, and should be determined for each new phage-host cell combination.

The size of the bacterial inoculum also affects the phage yield, the yield increasing with the inoculum to an optimum at about 2 x 10^9 cells per plate. The physiological state of the bacterial inoculum seems to have little influence on the phage yield, since the yields obtained using bacteria washed from an agar slant after overnight incubation did not differ from those obtained using broth grown bacteria in the logarithmic growth phase, providing the inocula contained equal numbers of cells. However, the yields obtained by using slants stored in the refrigerator for some days were decreased.

Variation in the incubation period of the inoculated plates also affected the phage yield, the optimum being about 10 hours at 37°C. Plates harvested after 4 hours' incubation contained large numbers of unlysed bacteria with a consequent reduced phage

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yield. The yield was reduced to 1/4 the maximum in plates harvested after 24 hours of incubation and further reduced to 1/10 the maximum after 48 hours. This loss is probably due mainly to diffusion of phage into the basal agar layer from which it is not recovered, although there may be some inactivation of the phage on prolonged incubation. The best time to harvest the plates is immediately after all host bacteria are lysed as judged by complete clearing of the plates.

Variation in the volume of base agar used per plate did not have an appreciable effect on phage yield over a range from 25 to 40 ml per plate, but a further reduction to 15 ml per plate did result in a decrease in yield. Omission of the base agar resulted in a virus yield no better than that obtained in fluid media. The layer of base agar serves as a source of nutrient for the dense population of bacteria growing in the soft agar layer.

It was thought that an increase in volume of soft agar in the agar layer might produce a larger yield per plate and thus make for a more efficient utilization of material. Consequently 2 sets of 5 plates were inoculated using 2.5 ml and 5 ml of soft agar per plate. The inocula for the first set were 1.1 x 10^5 T4r and 2 x 10^8 B per plate and for the second set twice these amounts. After incubation the agar layers were washed off using 5 ml of broth per plate for the first set and 10 ml for the second set. The recovered yields were 16 ml at 6 x 10^{10}/ml or 9.6 x 10^{11} total for the first set and 36 ml of 2 x 10^{10}/ml or 7.2 x 10^{11} total for the second set. Hence doubling the agar layer resulted not only in a decrease in the virus concentration but in a decrease in total yield per plate as well. This may have been due to a poorer nutritional state of the bacteria in the deeper soft agar layer.

The volume of broth used in extraction of phage from the agar layer is an obviously important factor in determining both the concentration and yield of phage obtainable. A number of plates were inoculated with 9 x 10^4 T4r and 1.5 x 10^8 B in 2.5 ml of soft agar. After overnight incubation groups of 3 plates were washed with varying amounts of broth and the pooled washings were assayed for virus after removal of the agar by centrifugation with the results seen in Table II.

TABLE II. Yield and Concentration of Recovered Virus as a Function of Volume of Broth Used for Washing Each Plate. Yields are based on pooled washings from 3 plates.

[illegible] ml/plate	Phage yield per ml	Broth recovered, ml	Phage recovered, total
2.5	1.3 × 10^{11}	7	9.1 × 10^{11}
5	9.6 × 10^{10}	15	1.4 × 10^{12}
10	4.6 × 10^{10}	29	[illegible]

Increasing the volume of broth from 5 to 10 ml per plate reduced the concentration of phage obtained but did not affect the total yield. Reducing the volume to 2.5 ml increased the concentration of phage obtained in the extract but decreased the yield of phage since a larger proportion of the total phage is lost in the sedimented agar. It would appear from this experiment that the maximum concentration of phage obtainable in the broth extract is limited by the phage concentration in the agar layer itself, which in this case appears to lie between 2 and 3 x 10^{11}/ml. The true phage concentration may be higher than this if there is any tendency for the phage to adsorb preferentially to the agar. The maximum recoverable phage was determined by repeatedly extracting the pooled agar layers from 5 plates using 25 ml of broth each time. The titers obtained in 7 successive extractions were 9.1 x 10^{10}, 2.8 x 10^{10}, 1.2 x 10^{10}, 6 x 10^9, 4.1 x 10^9, 2.6 x 10^9 and 1.8 x 10^9/ml. The total phage recovered thus amounts to 2.9 x 10^{11} particles per ml of agar layer, most of which was in the first two washings.

As a result of these studies it was possible to increase the concentration of phage T4r in plate washings from about 6 x 10^{10}/ml to 10^{11}/ml. However, this is still far less than the 10^{12}/ml readily obtainable with phage T5. This discrepancy in phage yields may in part be explained by the difference in burst sizes, that of T5 being 400 while that of T4r is only 125. If equal numbers of bacteria undergo lysis in the two cases and other factors are equivalent, the yield of T4r should be 1/3 that of T5 rather than 1/10.

Another factor which may affect the virus yield is the rate of adsorption of virus to host cell. Delbruck(7) concluded that the yield of virus particles in broth cultures would be increased by a decrease in adsorption rate for two reasons: the loss of liberated virus by multiple adsorption onto unlysed bacteria would be lessened, and the bacterial population would reach a higher total figure because delayed adsorption would give more time for bacterial growth. Since the adsorption rate of T4 is about 20 times that of T5, this difference may well contribute to the better yields obtainable with phage T5. The rate of adsorption of T4 is in part determined by the salt concentration in the medium(8). An attempt to further increase the yield of phage T4 by reducing the salt concentration to decrease the adsorption rate did not result in a significant gain in phage production.

Summary. Some of the variables involved in the production of high titer phage stocks by the agar layer method have been investigated using coli phage T4r as an example. The significant factors have been shown to be the number of virus particles and the number of bacteria inoculated per plate, the length of incubation, the volume of soft agar in the agar layer and the volume of broth used for extraction of the virus from the agar. By an appropriate adjustment of these variable factors it is possible to obtain stocks of the various coli phages of the T series ranging from 10^{11} to 10^{12} infectious particles/ml.

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Therapeutic Activity of Xanthopterin Added to p-Aminobenzoylglutamic Acid in Experimental Macrocytic Anemia of Rat.* (19077)

LEONARDO TENTORI AND GIROLAMO VIVALDI. (Introduced by A. J. Goldforb.)
From Istituto Superiore di Sanità, Roma, Italy.

We have reported(1) the results of experiments on rats in which macrocytic anemia was induced by a folic acid-deficient diet containing sulfaguanidine as an inhibitor of the intestinal flora. These animals were injected with the following substances separately: Folic acid, vitamin B_{12}, xanthopterin, cobalt ions and a mixture of xanthopterin and p-aminobenzoylglutamic acid. It was found that simultaneous administration of the last 2 drugs did not prevent the death of rats showing a severe vitamin deficiency. However, rats receiving streptomycin, instead of sulfaguanidine, and given the xanthopterin-paraminobenzoylglutamic acid mixture, showed a clear improvement of the blood picture.

In the present experiments we attempted to obtain a chronic folic acid deficiency in the rat by adding streptomycin, as an inhibitor of intestinal flora, to the usual synthetic diets. In addition the activity of xanthopterin, of p-aminobenzoylglutamic acid, and of both substances administered together was tested.

Experimental procedure. A total of 50 male albino rats, from this Institute strain, weighing 40 g each, were used. The animals were fed *ad libitum* with a standard folic acid-deficient diet(2). One percent of streptomycin was added to the diet as inhibitor of intestinal flora, instead of sulfasuxidine. The symptoms of deficiency were studied according to the technic previously described(1).

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* We are indebted to Lepetit Laboratories-Milano for supplying p-aminobenzoylglutamic acid, and to F. Hoffmann La Roche-Basel for xanthopterin used in these studies.

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TABLE I.*

[illegible]	R.B.C. × 10⁶ [illegible]	W.B.C. × 10³ [illegible]	[illegible]	C. I.
		Before treatment		
I	4.4 ± .42	8.5 ± 1.07	83 ± 9	1.52 ± .002
II	4.6 ± .63	8.8 ± 1	86 ± 9.6	1.40 ± .01
III	4.3 ± .05	8.9 ± .77	86 ± 4.80	1.67 ± .14
IV	4.4 ± .39	8.5 ± .34	94 ± 6.24	1.81 ± .01
		After treatment		
I	7.8 ± .22	13.6 ± 1.80	82 ± 2	.90 ± .001
II	5.3 ± .93	9.1 ± .77	91 ± 10	1.48 ± .01
III	5.7 ± .26	8.8 ± .94	88 ± 5.12	1.29 ± .09
IV	7.8 ± .02	11.2 ± .06	97 ± 9.05	1.07 ± .54

C.I. = Color index. Stand. error = $\sqrt{\frac{S\,y^2}{n(n-1)}}$.

* Emmens, C. W., Principles of Biological Assay, Chapman and Hall, London, 1948, p17.

Immediately after a period of depletion (about 180 days), the animals showed a hematological picture characteristic of folic acid deficiency. Twenty were selected and divided by random selection into 4 groups, on which the therapeutic activity of the following substances, administered parenterally (g wt. animal) was tested: Group 1, pteroylglutamic acid (folic acid), 200 µg; Group 2, p-aminobenzoylglutamic acid, 300 µg; Group 3, xanthopterin, 200 µg; Group 4, xanthopterin, 200 µg + p-aminobenzoylglutamic acid, 300 µg.

Results and discussion. The results are shown in Table I and Fig. 1 to 4. The deficient diet containing 1% streptomycin causes folic acid deficiency in about 50% of the animals. This technic has the following advantages: the animals show a more regular growth curve and a greater resistance to infection and the well known antithyroidal action of sulfa drugs is avoided. Rats fed on the streptomycin-containing diet develop a hematological picture equally characteristic but less severe than that shown by animals fed with diets containing sulfaguanidine. Erythrocyte levels range from 4 to 5 millions per mm. and the color index is above normal. The hematological data do not show significant variations even when observations were continued beyond 180 days. During this period definite evidence of vitamin deficiency appeared and persisted for 300 days.† Persistence of the anemia suggests that the animals were maintained in a chronic but partial deficiency state. Small amounts of folic acid may have been synthesized in the intestine in amounts which prevented severe bone marrow damage and death of the animals. It is possible, therefore, that streptomycin does not completely inhibit folic acid synthesis. Thus the addition of streptomycin to the diet seems to be particularly useful in the study of chronic folic acid deficiency in the rat. With this technic it is possible to avoid the periodic addition of suboptimal doses of folic acid to the diet as Kodiceck and Carpenter(3) have suggested. The experiment with the first group of rats treated with 200 µg of folic acid was performed to determine whether the hematological picture was due exclusively to the chronic deficiency of the vitamin. The results obtained (Fig. 1) confirm this hypothesis. We have used these results, therefore, in evaluating the activity of the other substances tested. The structural formulae of the substances used is given. It is evident that all of them contain a nucleus which appears also in the folic acid molecule.

The observations shown in Fig. 3 reveal (a) that administration of xanthopterin causes some increase in number of red blood cells, but is unable to restore the number to normal; (b) that the color index remains high while the leukocyte counts remain almost unchanged. Xanthopterin has very little activity,

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† Personal data unpublished.

3. Kodiceck and Carpenter, *Blood*, 1950, v6, 499.

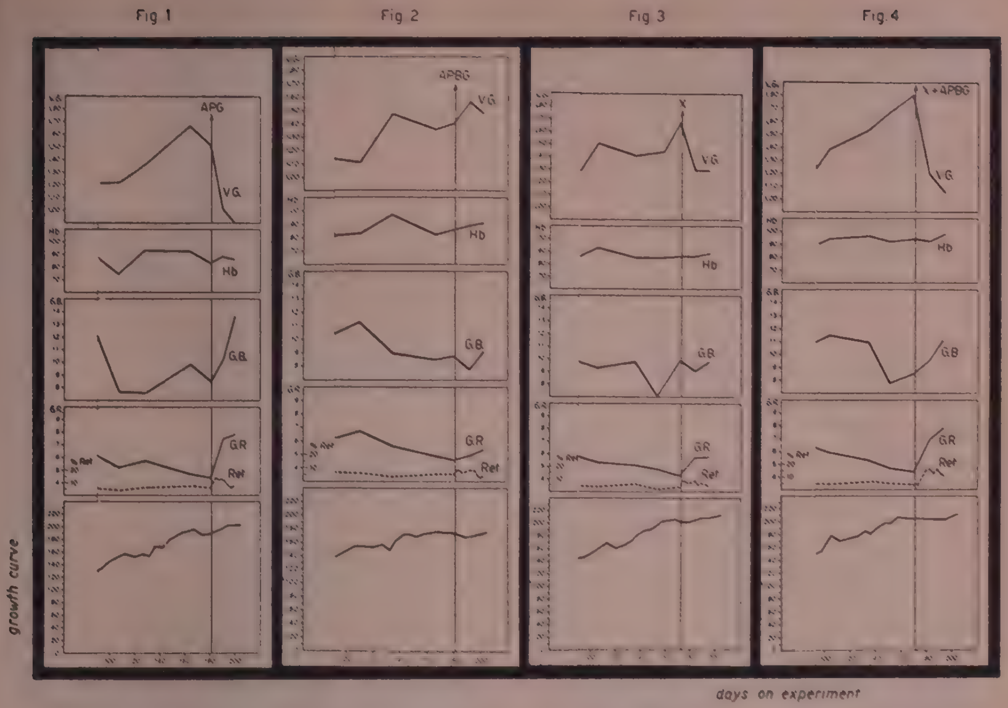
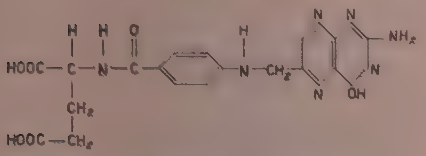
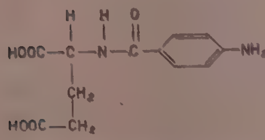


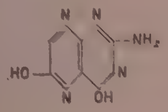
FIG. 1-4. The curves are plotted on the average data. Abbreviations: VG = color index; Hb = hemoglobin % (Sahli); Ret. = reticulocytes %; GB = white blood cells 10^3/cu mm; GR = red blood cells 10^6/cu mm; APG = pteroylglutamic acid; APBG = p-aminobenzoylglutamic acid; X = xanthopterin; X + APBG = xanthopterin + p-aminobenzoylglutamic acid.



Pteroylglutamic acid (folic acid).



P-aminobenzoylglutamic acid.



Xanthopterin.

therefore, causing some improvement in the anemia but not modifying its characteristic picture or altering the leukopenia. P-aminobenzoylglutamic acid had no activity (Fig. 2): the hematological findings were not altered by its administration. The mixture of xanthopterin-paraminobenzoylglutamic acid had complete folic acid-like activity. Although its action was somewhat slower than that of folic acid, the hematological picture eventually returned to normal (Fig. 4). Reticulocytosis occurred as with folic acid administration. While a reticulocyte crisis does not occur in animals treated with xanthopterin alone or with p-aminobenzoylglutamic acid alone, such a crisis appears in groups of animals receiving folic acid and the mixture of xanthopterin-paraminobenzoyl-glutamic acid. This observation suggests that these animals could perform folic acid synthesis if administered the two main components of its molecule.

Conclusions. (1) Chronic folic acid deficiency was induced in rats with a highly purified diet containing 1% streptomycin. (2) Xanthopterin causes a small increase in the number of erythrocytes but does not alter the color index or leukopenia. (3) P-aminobenzoylglutamic acid has no effect on the hematological picture resulting from a chronic deficiency of folic acid in the rat. (4) The simultaneous parenteral administration of amounts of xanthopterin and p-aminobenzoylglutamic acid in the same molecular ratio existing in folic acid, has a complete and specific therapeutic activity almost as rapid as that of folic acid. (5) This indicates that rats can synthesize folic acid from simultaneously administered xanthopterin and p-aminobenzoylglutamic acid.

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Changes in Brain Water, Brain Chloride, and Serum Chloride after Bilateral Nephrectomy in Rats. (19078)

ROBERT G. SMALL AND W. A. KREHL.

From Yale University Nutrition Laboratory, Department of Physiological Chemistry, New Haven, Conn.

In 11% of the 138 cases of cerebral edema reviewed by the authors from the autopsy files at the New Haven Hospital(1), nephritis and renal failure were found to be associated with edema of the brain. Other scattered references in the literature indicate that uremia and renal disease are often associated with cerebral swelling and edema(2-5). Hence one might expect to find edema in the brains of nephrectomized rats. The observations of Stewart-Wallace(6) suggest that chloride concentration, which he found much higher in edematous than in normal brain tissue, might serve as a biochemical index of cerebral edema. It is to be expected that in edema, in which there is an increase in the extracellar phase of a tissue, there should also be an increase in chloride, the predominant extracellular ion.

Although methods exist for the determination of tissue chlorides(7-9) preliminary experiments made it clear that a special procedure for chloride determination in small quantities of brain tissue is desirable. Enough normal and nephrectomized rats were used to justify statistical analysis of the results. Serum chloride and brain water determinations were done on both.

Experimental. Rats of the Sprague-Dawley and Yale strains were used. Bilateral nephrectomy was carried out under nembutal anesthesia and care was taken to leave the adrenal glands undisturbed. The animals were housed in an air conditioned animal room in which temperature and humidity were maintained at 75°C., and 45-55%, respectively. Purina laboratory feed and water were supplied *ad libitum*. The animals survived for 3 days after the operation, and just before death they were anesthetized with nembutal and a sample of blood was removed under oil by cardiac puncture. The skull was then removed with an electric hand saw, the pia-arachnoid was reflected, and the brain was lifted out with a spatula. The animals lived until the brain was removed. The blood covering the surface of the brain was removed by blotting with filter paper. The brain was then bisected and each half was weighed quickly. One half of the brain was used for the chloride determinations and the other for the water determinations.

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Method for brain chloride. Several modifications of the micro method of Van Slyke (8) were made to adapt it specifically for the measurement of the chloride in the brain. The following procedure, therefore, is presented as a suitable method for accurately measuring chloride in small samples of cerebral tissue. After careful weighing, each fresh half-brain (which weighs about 0.8 g) is homogenized in a Potter-Elvehjem homogenizer. During the process distilled water is added to increase the volume to 25 ml. Four 5-ml aliquots are pipetted into each of four 50-ml round-bottomed centrifuge tubes for parallel determinations on each sample. A measured amount of chloride is added to one of these aliquots as a control recovery determination. One ml of a standard nitric acid-silver nitrate reagent is then added to each aliquot, while the samples are briskly agitated. Four ml of concentrated nitric acid and a glass bead are then mixed with each sample in the centrifuge tube to provide an adequate volume for the digestion which is carried out over a bank of microburners until the solutions are a perfectly clear yellow-green color (20 min.). Decolorizing agents are not used. After digestion, 5 ml water and 0.3 g ferric ammonium sulfate indicator are added to the clear digest and 1 ml amyl alcohol is layered over the top. The amyl alcohol effectively sharpens the endpoint by concentrating the silver thiocyanate precipitate at the top of the titration mixture, making it possible to detect very slight color changes in the clear solution below. The titration is carried out after thoroughly cooling the tubes in an ice-water bath, using a microburette calibrated to 0.01 ml which delivers 150 drops per ml. The end point can be determined within 2-3 drops. The last drop brings a slight but definite color change (from yellow-green to orange) in the clear digest. All procedures were controlled, recovery determinations showing less than 1% error. When sufficient accuracy is attained, one aliquot can be used for each half-brain, eliminating the necessity for pipetting and thus decreasing the possibility of technical error. The values for brain chloride in the normal rats agree with those previously reported(9) in the literature. *Silver nitrate* (0.0521 N) is prepared in a darkroom by dissolving the weighed chemical in a minimum of distilled water and bringing the solution to volume with colorless concentrated nitric acid. Potassium thiocyanate (0.0224 N) and sodium chloride (0.0348 N) are prepared in the usual manner and standardized against the silver nitrate. Reagents of these concentrations are of the optimum strength for a clear end point and accurate readings on the microburrette in the analysis of chloride in small samples of brain tissue weighing 1 g or less. *Brain water concentration* is determined by drying weighed homogenized brain halves for 48 hours at 104°C. The homogenates are placed in small weighed flat aluminum pans in the drying oven. A large surface is exposed to the air and little water can be trapped in the small particles produced by homogenization. At 104°C., brain will show small decrements in weight even after a week or more of drying, but the decrease after 48 hours is very small and probably due to chemical changes, rather than water loss. *Serum chloride* determinations on the blood collected anaerobically under oil are carried out according to the procedure completely discussed by Peters and Van Slyke(10).

Results. The experimental values are presented in Table I. The "t" test of Student and the "f" test of Fisher(11, 12) were used to evaluate the results. An increase of 1.48 meq/kg wet weight of brain chloride was found in the nephrectomized animals. The variability of values in the nephrectomized group as shown by the "f" test makes this small increase even less significant. The nephrectomized animals also show a slight decrease of brain water which is significant at

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TABLE I.

	Normal			Nephrectomized			Statistical			
	No. of animals	Mean value	Standard deviation	No. of animals	Mean value	Standard deviation	"t" value	"p" value	"t" value	"p" value
Brain chloride, meq/kg, wet wt	19	33.7	1.39	20	35.18	3.28	1.775	10%	5.58	.01%
Serum chloride, meq/l	25	98.8	4.58	13	71.8	8.30	12.58	.001%	3.40	.01%
Brain water, % wet wt	42	78.2	.835	12	77.6	.692	2.237	5%	1.37	N.S.*

* N.S. = Not significant.

the 5% level.

Discussion. It can be seen (Table I) that while no significant change took place in brain water or brain chloride after nephrectomy, a substantial reduction in serum chloride occurred. This is in keeping with other observations that brain chloride concentration is extremely stable in spite of wide fluctuations in serum chloride(13-17). Since vomiting, diarrhea, and increased fluid intake were not observed in the nephrectomized animals, one cannot explain adequately the fall in serum chloride on the basis of external loss. If some of the water supplied *ad libitum* and the metabolic water was not lost via the lungs or skin, its retention might account for some of the serum dilution.

A fall of serum chloride has also been reported in dogs after bilateral nephrectomy(18, 19) and in man in chronic nephritis(20). Wilde(21) suggests that the accumulation of potassium after nephrectomy may carry chloride into tissue cells according to the Boyle-Conway theory(22,23), while Durlacher(24) showed that a low potassium diet prolonged the life of nephrectomized rats.

Edema of the brain did not occur in these nephrectomized rats nor was there any increase in brain chloride concentration. Eichelberger, Kollros, and Walker have chemically analyzed the brains of dogs subjected to cerebral concussion(7). Although cerebral edema has long been thought to occur in brain trauma, they found no chemical evidence of edema and no increase in chloride concentration in the traumatized brains. In similar experiments Yannet and Darrow(25) did find an increase in the chloride content of the brains of dogs subjected to hyperthermia. They assumed that edema was present since the increment of chloride, an extracellular ion for the most part, indicated an expansion of the extracellular phase of the brain tissue (edema). Our own and these experiments are an approach to the cerebral edema problem which, it is to be hoped, will be clarified by further biochemical investigation. It should of course be emphasized that the results reported here are based on an acute situation; whether they would also be obtained in a chronic situation remains to be determined.

Summary. (1) An accurate procedure is presented for the determination of chloride in small samples of cerebral tissue. (2) Cerebral edema did not occur in rats subjected to

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bilateral nephrectomy, nor was there any increase in brain chloride concentration. (3) After nephrectomy, the serum chloride of rats falls markedly.

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Utilization of d-Biotinol by Microorganisms, the Rat and Human.* (19079)

L. Drekter, J. Scheiner, E. De Ritter, and S. H. Rubin.

From the Nutrition Laboratories, Hoffmann-La Roche Inc., Nutley, N. J.

Following the synthesis of d-biotinol, the alcohol analog of d-biotin, by Sternbach, Kaiser and Goldberg(1), it was of interest to compare the biological activity of this derivative to that of biotin. Alcohol analogs of other vitamins are oxidized readily to the corresponding vitamin by animal organisms as shown by urinary excretion studies. For example, d-panthenol, the analog of d-pantothenic acid, and 3-pyridinemethanol, the analog of nicotinic acid, are converted efficiently by humans(2,3); panthenol has been shown to replace pantothenic acid in the nutrition of the rat(4,5). The experiments to be described concern the activity of biotinol for growth of microorganisms and the rat and include determinations of the excretion of biotin after administration of biotinol to the rat and human.

Experimental. (1) *Microbiological Activity.* The biotin activity of several biotinol preparations was determined for *Lactobacillus arabinosus*(6), *Lactobacillus casei* (7), and *Saccharomyces cerevisiae*(8), the organisms commonly used for assay of biotin. Each lot tested showed the same activity for all three organisms, but the activity varied from lot to lot, ranging from 0.2 to 2%. This variability suggested a probable contamination with small amounts of biotin. Separation of this biotin was achieved by passage of solutions containing 1 mg of biotinol per ml through a column of Amberlite IRA-400 as described by Weiss *et al.* for panthenol(9). Microbiological assays of the column eluates showed the biotin activity of biotinol *per se* to be less than 0.05%. This inactivity is analogous to the failure of panthenol to replace pantothenate in the nutrition of *Lactobacillus arabinosus and Saccharomyces carlsbergensis* (2). Recovery tests with 2% and 20% biotin added to the biotinol solution indicated complete removal of the added biotin by the resin. Quantitative recovery of the biotinol in the column eluate was demonstrated by rat excretion assays in which equal urinary excretions of biotin were found after oral dosage of resin-treated and untreated biotinol solutions.

(2) *Rat curative assay.* Biotinol was compared to biotin as regards activity in curing egg white-induced biotin deficiency. The 30% egg white diet, vitamin supplement and test conditions were the same as those reported by Rubin, Drekter and Moyer(10).

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TABLE I. Wt Gain of Biotin-Deficient Rats Fed d-Biotin or d-Biotinol.

Supplement	Dose (fed 3 times weekly), μg	No. of rats	Avg gain in 4 wk, g ±S.E.
None	—	3*	3 —
Biotin	.80	5	44 ± 4.3
Biotinol	.75	5	41 ± 7.3
Biotin	1.60	5	62 ± 6.2
Biotinol	1.50	5	85 ± 5.6

* 1 rat died.

Weight gains in the 4-week assay period are given in Table I and show biotinol to be as effective as biotin in promoting growth of these biotin-deficient rats. The fact that the response is greater with biotinol than with biotin at the 1.6 mcg level is not considered significant in view of the small number of rats involved and the individual variations found. Accompanying the weight gains of the test animals was a disappearance of the dermatitis and alopecia with both biotinol and biotin.

(3) *Rat Excretion Studies.* (a) *Basal Excretions.* Basal urinary and fecal excretions of biotin by 200-300 g rats on a Friskie dog chow diet were determined by assay with *L. arabinosus*(6). The average urinary excretion was 1.3 μg per day ± S.D. 0.45. Direct assays of the feces averaged 4.5 μg per day, but after autoclaving with sulfuric acid for 2 hours at 15 lb, the fecal biotin assay increased to 10 μg per day. No difference was found when 1, 2 or 4 N sulfuric acid was used for this hydrolysis.

(b) *Urinary Excretions after Low Doses* (Table II). After oral dosage by catheter of 50 μg or less of biotin or equivalent amounts of biotinol, 25 to 38% of the dose is excreted as biotin in the urine in 24 hours. The lack of significant difference between biotinol and biotin at the 3 levels fed indicates an efficient oxidation of biotinol to biotin by the rat.

After intramuscular injection of 50 and 158 μg of biotin or equivalent amounts of biotinol, the urinary excretion of biotin is greater after biotin than after biotinol (Table II), but at the 225 μg level there is no difference. Since only 4 rats were tested in a group at the 2 lower levels and the urines pooled for assay, the significance of the difference after biotin and biotinol cannot be evaluated. The equivalent excretions at the 225 μg level where more rats were tested indicates an efficient conversion of biotinol to biotin after intramuscular injection.

(c) *Urinary and Fecal Excretion after High Doses* (Table II). After oral dosage of 3.4 and 10 mg, a much higher percentage of the dose appears in the urine after biotinol than after biotin. In the latter case the fecal excretion of biotin is considerably greater than after biotinol so that the total of urinary and fecal excretions is only slightly lower after biotin. This difference in excretion may well arise from the fact that the solubility of biotinol in water is at least 5 times as great as that of biotin. This effect is not shown after intramuscular injection where the urinary excretion of the biotin is higher than after oral dosage, and of the same order of magnitude as after intramuscular biotinol.

(4) *Human Excretion Studies.* A preliminary investigation was made of the extra urinary biotin excretion after oral or intramuscular administration of biotin and biotinol. The subject was a 33 months old male whose urinary biotin excretion had returned close to the basal level of 4 μg per day after previous intake of biotin. One mg of biotin or biotinol was given daily for 2 days followed by 2 days without supplementation. In the first 2 test periods biotinol was given orally and then intramuscularly, and in the next 2 test periods the same procedure was followed with biotin. After oral administration, 20% and 48% of the two biotin doses and 51% and 59% of the biotinol doses were excreted in the urine as biotin in 24 hours as determined by *L. arabinosus* assay. After intramuscular injection, 54% and 87% were excreted in the 24-hour urines after biotin and 54% and 47% after biotinol. Although the limited nature of the data and the fluctuations in excretions preclude a quantitative estimation of the relative utilization of biotin and biotinol, it is evident that biotinol is converted rather efficiently to biotin by the human after both oral and intramuscular administration.

Summary. d-Biotinol does not replace d-biotin in the nutrition of *L. arabinosus*, *L. casei*, or *S. cerevisiae* but is fully as effective

TABLE II. Urinary and Fecal Excretion of Biotin After Administration of Biotin or Biotinol to Rats.

Dose (as d-biotin)	No. of rats		Extra excretion in 24 hr — d-Biotin		Extra excretion in 24 hr — d-Biotinol	
				% of dose		% of dose
			Oral			
µg			µg		µg	
10	4	Urine	2.9	29	3.7	37
25	8	”	6.3	25	6.8	27
50	16–18	”	19	38	17	34
mg			mg			
3.4	2	”	1.6	47	3	88
		Feces	1.4	41	.14	4
10	6	Urine	2.2	22	7.2	72
		Feces	5	50	1.1	11
			Intramuscular			
µg			µg			
50	4	Urine	22	44	11.4	23
158	4	”	83	53	62	39
225	12–14	”	112	50	113	50
mg			mg			
5	4	”	3.5	70	2.8	56

as d-biotin in curing egg white-induced biotin deficiency in the rat. Comparison of urinary excretions of biotin after oral or intramuscular administration of biotin and biotinol shows the latter to be converted to biotin efficiently by both rat and human.

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Absence of Analgesia During Induced Hyperadrenalism.* (19080)

DE GUISE VAILLANCOURT,† ALBERT W. GROKOEST, AND CHARLES RAGAN.

From the Department of Medicine, Columbia University College of Physicians and Surgeons, and the Edward Daniels Faulkner Arthritis Clinic of the Presbyterian Hospital, New York.

Selye first ascribed anesthetic properties to hormonally active steroids(1-3) and others (4,5) have suggested that cortisone and ACTH produce analgesia if not anesthesia. Thus, we examined the threshold for pain in patients with rheumatoid arthritis before and during therapy with cortisone and ACTH.

There are probably two major types of superficial or cutaneous pain(6), both of which we have attempted to evaluate. Two categories of visceral pain have also been described, the so-called "true visceral pain," a diffuse poorly localized sensation, and

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[†] Fellow of the Canadian Arthritis and Rheumatism Society.

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"referred pain" perceived in an area away from the site of the stimulus(7). In our experiments, we have studied only "true visceral pain."

Method. The threshold to superficial and visceral pain was determined in a group of patients with rheumatoid arthritis during a control period. The same stimuli were repeated after the patients had been treated with 700-900 mg of cortisone in a 6-day period or 240 mg ACTH in a 4-day period, until a remission of symptoms or the usual clinical and laboratory manifestations of hyperadrenalism appeared. "Superficial pain" was elicited by injections of 0.1 cc saline and distilled water intradermally, by the application of radiant heat, and by electrical stimulation of the dental pulp and peridental membrane. Histamine injections, as suggested by Rosenthal(8) proved to be unreliable. Saline at a neutral pH produced a barely noticeable sensation insufficient for our purposes but distilled water was more painful and the results could be quantitated when compared with pain previously experienced; the slightest discomfort being 1+, the maximal pain 10+. 0.1 cc of distilled water intradermally generally provided similar "estimates" of the intensity of pain on repeated determinations. The stimulus threshold of the dental pulp and the peridental membrane was measured electrically by the method of Ziskin and Zegarelli(9). Visceral pain was elicited by stretching the second or third portion of the duodenum by air injected into a rubber balloon fixed to the distal end of a Miller-Abbott tube, as suggested by Polland and Bloomfield(10). The threshold for "true visceral pain" was reached when the patient first stated that a new sensation was felt in the abdomen. The volume of air in cc in the balloon and the balloon pressure in mm of Hg were then recorded. At the threshold level, the patients described the location, the quality and the intensity of the sensation experienced. The pain threshold in a series of 5 patients was established before and during hormonal therapy, using the Hardy, Wolff, Goodell technic(6,11,12). The method of D'Amour and Smith(13) was used to evaluate pain sensation in rats. A light intensity which produced a reaction in about 5 seconds was most convenient and the method was simple with excellent reproducibility of results. Rats weighing from 120 to 182 g received subcutaneous injections of 10 mg of cortisone acetate daily for 4 days; readings were taken before hormone administration and on the fourth day of injection.

Results. In all instances, the pain threshold for cutaneous dental or visceral (Table I) sensation was not significantly influenced by either cortisone or ACTH at the usual therapeutic dose levels. Similarly, cortisone failed to produce a measurable degree of analgesia in markedly hyperadrenal rats (Table I).

The average mean threshold obtained by electrical stimulation applied to 5 teeth in each patient showed no significant change in sensitivity during hormonal therapy.

There was no increase in the visceral pain threshold to balloon distention during hormonal therapy in the 6 patients tested (Table I), while after a 30 mg injection of morphine a 3-fold increase in volume of air and a 2-fold increase in pressure were required to reach the visceral pain threshold.

Discussion. In our experience, the administration of cortisone or ACTH to humans in doses adequate to control the symptoms of rheumatoid arthritis produced no measurable anesthetic or analgesic effect.

The alleviation of pain observed in clinical syndromes treated with either cortisone or

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TABLE I. Cortisone and ACTH on Various Types of Induced Pain.

Type of pain stimulus	Subject	Hormone	Before hormone (range)	During hormone (range of change)
I Cutaneous pain*	5 patients	4 cortisone, 1 ACTH	1+ to 3+	0 to —1+ **
” ” †	5 ”	4 ” 1 ”	7+ to 8+	—1+ to +1+ **
II Pain threshold‡	5 ”	Cortisone	157 to 232 mc	—10 to +8 mc ††
III Visceral pain	6 ”	4 cortisone, 2 ACTH	40 to 75 §	—20 to 0 § ††
	1 ”	Morphine sulfate	50 §	+100 § ††
	4 ”	3 cortisone, 1 ACTH	1.7 to 3 ‖	—.7 to +.4 ‖ ††
	1 ”	Morphine sulfate	1.7 ‖	+1 ‖ ††
IV Pain from radiant heat	5 rats	Cortisone	6.2 to 8.6¶	0 to +1.4 ¶ ††

Stand. dev. were calculated for all hormone experiments and in no instance was the change found statistically significant.

* Intradermal NaCl. † Intradermal distilled water. ‡ As measured by Wolff-Hardy-Goodell apparatus using dorsum of hand. § Volume of air in balloon in cc required to produce a sensation. ‖ Pressure of air in balloon in cm Hg required to produce a sensation. ¶ Seconds. ** Decrease denotes analgesic effect. †† Increase denotes analgesic effect.

ACTH is probably due to an hormonally-induced alteration in the response of the host to the inflammatory reaction(14,15) or to a parallel decrease in the inflammatory reaction not necessarily affecting the etiologic agent(16).

Summary. A systematic evaluation of cutaneous, dental and visceral pain before and during the administration of either cortisone or ACTH demonstrated that these agents had no analgesic effect at therapeutic dose levels. The marked alteration in the host reaction to inflammation during treatment with cortisone or ACTH probably is the principal factor responsible for the alleviation of pain.

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Decreased Resistance of Pyridoxine-Deficient Rats to Cold Exposure.*

(19081)

BENJAMIN H. ERSHOFF.

From the Emory W. Thurston Laboratories, Los Angeles, Calif.

It is well established that the pituitary-adrenal system is activated under conditions of stress(1,2), and that requirements for ACTH(3) and adrenal cortical hormone(s) (4,5) are increased during adjustment to cold. Hypophysectomy (with the consequent reduction in ACTH secretion) and adrenalectomy (with the resultant decrease in adrenal cortical hormones) both impair adjustments to cold(4,6). A deficiency of thyroid hormone caused by thyroidectomy(7) or the administration of antithyroid drugs(8) also impairs survival under conditions of low environmental temperature. Various dietary factors including ascorbic acid(9), vitamin A(10) and riboflavin(11) are also essential for optimal adjustment to cold. In the present communication data are presented on the effects of pyridoxine deficiency on resistance to cold stress in the rat.

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Procedure and results. The basal ration employed in the present experiment consisted of sucrose, 60%; casein[†], 25%; salt mixture[‡], 5%; and cottonseed oil (Wesson), 10%. To each kg of the above diet were added the following synthetic vitamins: thiamine hydrochloride, 40 mg; riboflavin, 40 mg; pyridoxine hydrochloride, 40 mg; calcium pantothenate, 80 mg; nicotinic acid, 60 mg; ascorbic acid, 200 mg; biotin, 5 mg; folic acid, 10 mg; p-aminobenzoic acid, 400 mg; inositol, 800 mg; vit. B_{12}, 150 μg; 2-methyl-naphthoquinone, 10 mg; and choline chloride, 2 g. Each rat also received 3 times weekly a vit A-D concentrate[§] containing 50 U.S.P. units of vit A and 5 U.S.P. units of vit D, and once weekly 4.5 mg of alpha-tocopherol acetate. Tests were conducted(1) with rats fed the basal ration and (2) with animals fed a similar diet with the pyridoxine hydrochloride omitted.

Exp 1. Fifty-two female rats of the Long-Evans strain were selected at 25 to 28 days of age and an average weight of 56.0 g and fed the diets indicated above for 100 days, or until death. Tests were conducted (1) with animals kept continuously in a large walk-in refrigerator at a temperature of 2 ± 1.5°C and (2) under standard laboratory conditions at an average temperature of approximately 23 ± 2°C. Rats were kept in metal cages with raised screen bottoms to prevent access to feces and were fed the above diets *ad lib* (16 rats per group in the cold room series; 10 animals per group in the room temperature series). Diets were made up weekly and stored under refrigeration when not in use. Animals were fed on alternate days. All food not consumed 48 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet.

Findings indicate that under room temperature conditions all rats tested survived an experimental period of 100 days. This occurred on both the basal and pyridoxine-free diet. Gain in body weight was significantly greater on the control than the deficient diet, but animals in both groups gained weight continuously throughout the experimental period. No dermatitis occurred in any of the rats fed the pyridoxine deficient diet and with the exception of body weight no significant differences were observed grossly between the 2 groups. After 100 days of feeding the average gain in body weight of rats on the basal ration was 176.0 ± 4.2 g,[||] and the average weight increment of rats fed the pyridoxine deficient diet was 99.2 ± 5.1 g.[||] In the cold room series growth was retarded in all rats. Thirteen out of 16 (81.3%) of the

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[†] Vitamin Test Casein, General Biochemicals, Chagrin Falls, O.

[‡] Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Chagrin Falls, O.

[§] Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vit. A and 80,000 U.S.P. units of vit. D per g.

[||] Including standard error of the mean calculated as follows:

$$\sqrt{\frac{\Sigma d^2}{n}} \Big/ \sqrt{n}$$

where "d" is the deviation from the mean and "n" is the number of observations.

rats fed the basal ration survived the experimental period of 100 days with a weight increment of 122.6 ± 6.5 g;‖ 12 out of 16 rats (75.0%) fed the pyridoxine-deficient diet also survived with a growth increment of 64.4 ± 2.8 g.‖ As in the case of the room temperature series animals continued to gain weight continuously throughout the experimental period and no dermatitis or other manifestations of pyridoxine deficiency (with the exception of growth retardation) was observed grossly in any of the rats fed the pyridoxine-deficient diet.

Exp. 2. Fifty-two female rats of the Long-Evans strain 23 to 25 days old and averaging 46.2 g in weight were divided into 2 groups of 26 rats each and fed *ad lib* under standard laboratory conditions for a period of 80 days the basal ration and pyridoxine-deficient diets indicated above. After 80 days of feeding the average weight increment of rats fed the basal ration was 172.8 ± 5.4 g‖ and of animals fed the pyridoxine-deficient diet, 97.7 ± 6.3 g.‖ At this time each group was divided into 2 parts. Sixteen rats in each group were placed in a cold room maintained at a temperature of 2 ± 1.5°C; the remaining 10 animals in each group were continued at standard laboratory conditions (23° C). No change was made in the diets fed. Feeding was continued for an additional 20 days or until death, whichever occurred sooner. In the room temperature series all rats survived the additional 20 days of feeding with a weight increment of 17.9 g for animals on the basal ration and 6.3 g for rats fed the pyridoxine-deficient diet. In the cold room series, however, 11 out of the 16 rats fed the pyridoxine-deficient diet (68.8%) died within the 20 day period (average survival time 12.6 days); all rats fed the basal ration survived. The average loss in body weight during cold exposure was 12.4 g for rats on the basal ration and 33.5 g for surviving animals on the pyridoxine-deficient diet.

Discussion. Present findings indicate that rats raised to maturity on a pyridoxine-deficient diet readily succumb following cold exposure but continue to survive on a similar diet under room temperature conditions. The cause of death in such animals is apparently not due to an accentuation of the pyridoxine deficiency as a result of cold exposure but rather to a failure in the mechanism of adjustment to cold. This is indicated by the fact that no significant reduction occurred in the survival of rats fed a pyridoxine-deficient diet when such animals were placed in the cold prior to the time they were pyridoxine-deficient. It would appear, therefore, that pyridoxine is essential for optimal adjustment to cold but that dietary sources of this vitamin are dispensable for survival (at least in the rat) once adjustment to cold has occurred.

In contrast to earlier investigations under room temperature conditions in which rats fed a diet deficient in pyridoxine plateaued in body weight, developed dermatitis and died, animals fed a pyridoxine-deficient ration under conditions of the present experiment gained weight continuously and showed no gross manifestations of pyridoxine deficiency with the exception of retardation in growth. The cause for this discrepancy is not readily apparent. It is not unlikely, however, that the higher B vitamin and fat content in the present experiment stimulated a greater intestinal synthesis of pyridoxine or promoted a better utilization of any available pyridoxine-active nutrients.

Available data do not indicate what factors were responsible for the impaired adjustment of pyridoxine-deficient rats to a low environmental temperature. In preliminary studies(12) no significant difference was observed between rats fed a pyridoxine-deficient diet and animals fed a similar ration supplemented with pyridoxine hydrochloride in the leukocyte picture after epinephrine administration. Both groups showed a reversal in the differential count, an increase in total leukocytes, a marked increase in polymorphonuclear leucocytes and a significant reduction in lymphocytes and eosinophils 3 hours after epinephrine administration. These responses are indicative of an activated pituitary-adrenal system and are similar to those which occur in the normal animal after ACTH or

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12. Parrott, F. D., Jr., and Ershoff, B. H., unpublished data.

cortisone administration or in response to nonspecific stress(13,14). Twenty-four hours after the injection of epinephrine the blood picture of both pyridoxine-deficient and control rats had returned to pre-injection levels in respect to all cellular constituents with the exception of the eosinophils which were still reduced by over 50%. These findings indicate that pyridoxine deficiency does not impair the ability of the pituitary-adrenal system to respond to acute stress. The activation of the adrenals which occurs in response to epinephrine administration, however, is based primarily if not entirely on the release of preformed ACTH. It is possible that the pyridoxine-deficient rat may have sufficient amounts of this hormone stored in its pituitary to effect a normal response to acute stress but that such rats may have an impaired ability to produce the increased amounts of ACTH required in chronic stress. The role of pyridoxine in protein metabolism suggests the possibility that animals deficient in this vitamin may have an impaired ability to synthesize proteins including adrenocorticotropin, thyrotropin, and others. In view of the increased requirement for thyrotropin(15) and adrenocorticotropin(3) during prolonged exposure to cold, the failure of pyridoxine-deficient rats to adjust to low environmental temperature may result not from pyridoxine deficiency *per se* but rather from a deficient secretion of pituitary hormones. Further experiments are indicated to determine the effects of thyroid, ACTH and cortisone administration on the survival of pyridoxine-deficient rats under conditions of low environmental temperature.

Summary. Immature rats were fed a purified ration containing all the known B vitamins in synthetic form and a similar diet with pyridoxine omitted. Tests were conducted in which rats fed the above diets were exposed to cold (2 ± 1.5°C) from the first day of feeding or after an experimental period of 80 days. 68.8% of the pyridoxine-deficient rats in the latter group succumbed during a 20 day period of cold exposure in contrast to a 0% mortality for animals fed a similar diet supplemented with pyridoxine hydrochloride. In the group exposed to cold from the first day of feeding mortality was 25.0% and 18.7% respectively for rats fed the pyridoxine-deficient and control ration for a period of 100 days.

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Preparation of Specific Complement-Fixing Antigen with Lansing Poliomyelitis Virus.* (19082)

MORRIS POLLARD.
From the University of Texas Medical Branch, Galveston, Texas.

The complement fixation (CF) technic has been successfully applied to the study of many of the human viral encephalitides with a notable exception of poliomyelitis. Recently Casals and Olitsky(1) described the preparation and use of a specific CF antigen prepared from MEF$_1$ virus adapted to new-born mice. Pollard(2) described the preparation of a CF antigen from Lansing infected cotton rat (CR) tissues by precipitation of the virus with methanol and use of the eluate as antigen. A relatively rapid rise and fall in CF antibodies in immunized cotton rats was described with this antigen.

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* Supported by a grant from the National Foundation for Infantile Paralysis, Inc. Part of this problem was performed while at the Hooper Foundation for Medical Research, University of California Medical Center, San Francisco, Calif.

1. Casals, J. and Olitsky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1951, v75, 315.

2. Pollard, M., *J. Immunol.*, 1951, in press.

Loring, *et al.*(3) outlined a method of preparing a CF antigen by high speed centrifugation of the virus with the resuspended purified viral sediment being used as CF antigen. The technic recommended involved repeated saline extractions of the tissue and repeated cycles of centrifugation. For some reason the antigens thus prepared appeared to be somewhat nonspecific at low dilution and antigens from normal CR tissues also fixed complement with poliomyelitis serum.

By omitting many of the details advocated by Loring *et al.*, CF antigens were prepared by us in the ultracentrifuge which were highly specific. CF antigens were thus prepared from CR tissues infected with Lansing poliomyelitis, rabies (CVS strain), and St. Louis encephalitis viruses. Each CF antigen thus prepared acted as a control for the specificity of the others.

Methods. 1. *Preparation of Antigen.* Antigens were prepared from cotton rat nerve tissues infected with Lansing poliomyelitis (LCR), St. Louis encephalitis (SLE), and rabies (CVS strain) viruses, as follows:

a. Ultracentrifugation technic. The nerve tissue was emulsified in a Waring blendor to a final concentration in physiological salt solution of 20%. After clarifying the suspension at 5,000 rpm for 30 minutes and 18,000 rpm for 10 minutes, the clear liquid was thoroughly mixed with 1/3 volume of ether and stored at 4°C for 1 hour. After centrifugation at 5,000 rpm for 20 minutes, the aqueous portion was stored at —20°C overnight. The thawed preparation was then centrifuged at 5,000 rpm for 20 minutes and at 144,000 x G for 2 hours (Spinco Preparative Centrifuge[†]). The viral sediment was resuspended to 1/10 volume with neutral M/5 phosphate buffer, stored at 4°C overnight and then centrifuged at 5,000 rpm for 20 minutes. The clear supernatant fluid represented the antigen. Decimal dilutions thereof were inoculated into mature cotton rats (0.05 ml intracerebrally) in order to determine the quantity of viable virus present in the antigen. The antigen was stored at —20°C.

b. Lyophilization technic. 20% emulsions of nerve tissues in physiological saline were shell frozen in a dry ice-alcohol bath and immediately desiccated in a freeze-drying Cryochem apparatus.[‡] The dried tissue suspensions were then extracted 3 times with 3 volumes of C.P. benzene at hourly intervals. After removal of residual benzene with a vacuum pump, the dried material was resuspended to 50% concentration with distilled water. After hydration overnight at 4°C, the suspensions were centrifuged at 10,000 rpm for 15 minutes. The clear supernatant CF antigen was tested for virus by inoculation of decimal dilutions thereof into cotton rats and for complement fixing properties as described below.

2. *Antiserum.* Adult cotton rats were given 3 intraperitoneal inoculations of 10,000 cotton rat LD_{50} of Lansing and of MEF_1 viruses at weekly intervals. They were bled one week after the third inoculation and their pooled serum was stored at —20°C. The monkey serum employed was obtained from hyperimmunized *Macaccus mulatta* monkeys. Each monkey was bled and inoculated at weekly intervals into the gastrocnemius muscle with 1,000 monkey LD_{50} of virus mixed with pendil.[§] The clear serums were stored at —20°C.

3. *The CF test.* 0.2 ml quantities of antigen, inactivated antiserum, and complement (2 units) were mixed and after incubation overnight at 4°C, amboceptor and 2% sheep erythrocytes were added. The results were read after incubation at 37°C for 30 minutes. Cotton rat serums were diluted 1:5 and 1:10 in salt solution and inactivated at 61°C for 30 minutes; monkey serums, diluted 1:5, at 61°C for 20 minutes. Antigen, serum and

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3. Loring, H. S., Raffel, S., and Anderson, J. C., PROC. SOC. EXP. BIOL. AND MED., 1946, v66, 385.

[†] Specialized Instruments Co., Belmont, Calif.

[‡] Purchased from Stokes Machine Co., Philadelphia, Pa.

[§] "Pendil" contains oxycholesterol-derivative, peanut oil, and beeswax and is manufactured by Endo Products, Inc., Richmond Hill, N. Y.

TABLE I. "Box-Titration" of Lansing Poliomyelitis Complement Fixing Antigen Prepared by Ultracentrifugation of the Virus.

Serum dilution	Antigen dilution					
	1–0	1–1	1–2	1–4	1–8	1–16
1–5	4+	4+	4+	2+	—	
1–10	4+	4+	—	—	—	
1–20	4+	4+	—	—	—	
1–40	4+	2+				
1–80	4+					
1–160	—	—	—	—	—	

TABLE II. The Specificity of Complement Fixing Antigens Prepared by Ultracentrifugation of the Virus.

Cotton rat antiserums	Cotton rat antigens		
	LCR	Rabies	SLE
LCR	4+ 1:80	—	—
Rabies	—	4+ 1:40	—
SLE	—	—	4+ 1:80

TABLE III. Complement Fixation Reactions with Ultracentrifuged Antigens and Antiserum Prepared from Monkeys and Cotton Rats.

Serum	Cotton rat antigens		
	Lansing	Rabies	SLE
Lansing	4+ 1:40	Neg	Neg
Brunhilde	Neg	Neg	Neg
Leon	Neg	Neg	Neg
SLE*	Neg	Neg	4+ 1:80
Rabies*	Neg	4+ 1:40	Neg
LCR*	4+ 1:80	Neg	Neg

* Antiserum prepared in cotton rats. SLE = St. Louis encephalitis; LCR = Lansing cotton rat.

complement controls accompanied each test.

Results. Tables I and II indicate that specific CF antigens were prepared by the centrifugation technic described above. The Lansing antigens were not stronger than those prepared by other methods(1,2), rarely exceeding 1:2 dilution for one unit of antigen, but occasionally some antigens were used at 1:8 dilution. Most of the tests contained 2 units of Lansing antigen at 1:1 dilution. Rabies and SLE antigens contained 2 units of CF antigen in dilutions as high as 1:20 and 1:40; however, they were diluted 1:1 and 1:2 when used for control purposes. Serums from immunized cotton rats fixed complement only with the antigen against which they had been immunized. It appeared that the titer of CF antibodies for Lansing virus in cotton rats exhibited a chronological curve similar to that demonstrated previously(2): a sharp rise and rapid decline of CF antibodies. Lansing CF antibody appeared within 10 days after initial inoculation and became undetectable 3 months later. Serum from MEF_1-immune cotton rats fixed complement specifically with the Lansing antigen. The chronological rise and fall of cotton rat CF antibodies against rabies and St. Louis encephalitis viruses were not studied.

Serum specimens from 6 Lansing-immune monkeys fixed complement with Lansing antigen specifically (Table III); while with the same antigen, 3 serums from Brunhilde-immune monkeys and one from a Leon-immune monkey¶ were negative. Three monkeys which were inoculated intramuscularly with Lansing virus in "Pendil" developed CF antibodies in 10 days which persisted for 3 months thereafter. The serums of these monkeys were negative with rabies and SLE antigens.

Of 8 Lansing antigens prepared by the above technic, 7 were specific CF antigens. 10^{-4} dilutions of the latter 7 were infective for cotton rats by the intracerebral route; the other one was not. When ether treatment was omitted, the resulting CF antigen was nonspecific. Three CF antigens were prepared each with rabies and St. Louis encephalitis viruses and all were specific. The average yield of Lansing CF antigen per g of tissue was 0.5 ml. The Lansing antigen retained its CF properties for at least one month at —20°C.

When infected tissues were processed by the lyophilization technic described above, CF antigen could not be obtained. Consistently, the virus content of the antigens was 2-3 logs lower than that of the unprocessed emulsion. Six Lansing CF antigens were processed by the lyophilization technic and none was satisfactory. Specific potent CF antigens were consistently processed from SLE and rabies infected tissues by this technic.

Discussion. The Lansing CF antigens prepared in the ultracentrifuge were as potent as any previously described. The technic was considerably simplified and the results were uniformly satisfactory. Considering the cost of equipment and the yield of antigen per g of tissue (0.5 ml/g tissue), this antigen is no less expensive to prepare than that produced by methanol precipitation of the virus. Its advantage lies in consistent results and in the specific nature of its reaction.

¶ Kindly supplied by Dr. Jonas Salk, University of Pittsburgh, Pittsburgh, Pa.

It is now felt that the preparation of specific Lansing antigen is predicated on a high level of virus in the tissues employed. The one antigen which was unsuitable was infective only at 10^{-2} dilution.

Antigens prepared by procedures involving lyophilization have been neither antigenic nor infectious. The desiccation technics are very useful for the preparation of rabies and SLE antigens as has previously been demonstrated(4-6), but the technics here successful, failed with poliomyelitis virus. If the MEF_1 virus adapted to newborn mice by Olitsky and Casals(1) has developed a tolerance for desiccation, then these technics may be very useful for this strain of virus.

The Lansing CF antigen fixed complement with serum from Lansing-immune monkeys and failed to do so with serum from 3 Brunhilde-immune and one Leon-immune monkeys. The maximum titer of the Lansing serum was 1:40. Lansing antigens previously prepared with methanol(2) were negative with serum from immunized monkeys. This may be due to the more potent CF antibody response to the Lansing-"Pendil" inoculum or, perhaps, to the production of a more potent or complete CF antigen by the ultracentrifugation technic. The antibody enhancing response of monkeys inoculated with virus-adjuvant mixtures has been demonstrated by others(7,8). The persistent CF response in the Lansing-immune monkeys (3 months) might be attributed to the effect of the depot plus virus inoculum.

There is some evidence of strain specificity with the ultracentrifuged antigens: Lansing CF antigens fixed only with Lansing and MEF_1 antiserum. Since we have not yet demonstrated specific CF antibodies with the Brunhilde- and Leon-immune serums, the specific response must be regarded with some reservation. Specific CF antigens can be prepared for rabies and SLE viruses by technics involving the extraction of desiccated tissue suspensions with ether and benzene (4-6). It appears, however, that lyophilization is so destructive to poliomyelitis virus that the above technic is inapplicable to the conventional Lansing virus in cotton rat tissues(9,10).

The rise and fall of Lansing CF antibody in immunized cotton rats is not inconsistent with that previously encountered with other viral agents in humans and in animals(11,12). The persistent response in monkeys may be abnormal, since monkeys inoculated with virus in saline have manifested a very transitory CF response. If a transient serological pattern can be demonstrated in human infections, in specific manner for all types of poliomyelitis virus, it would be of much help in deciding whether the infection was current or past.

Summary. Specific complement-fixing antigens were prepared from cotton rat tissues infected with Lansing poliomyelitis, rabies, and St. Louis encephalitis viruses. The antigens were prepared by concentration of the viruses through high speed centrifugation. Lansing antigens prepared from lyophilized tissues were of little value as CF antigens.

A relatively rapid rise and fall in poliomyelitis complement-fixing antibody levels in cotton rats is described. The Lansing antigen fixed complement with serum from Lansing-immune monkeys, but not with serum from

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monkeys similarly immunized against Brunhilde and Leon viruses.

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Action of Adrenal Hormones on Lethal Toxicities of Certain Organic Compounds. (19083)

Lyle V. Beck.*

From the Department of Physiology and Pharmacology, School of Medicine, University of Pittsburgh, Pittsburgh, Pa.

It has been reported(1) that mice bearing Sarcoma 37 will tolerate larger doses of a number of tumor-damaging trivalent arsenical compounds than will non-tumor-bearing mice. The report by Dalton(2) that tumor growth in mice is accompanied by histologic changes in the adrenals suggested that the observed increased resistance of mice to trivalent arsenical compounds with growth of Sarcoma 37 might be related to a change in adrenal activity. This paper reports results of experiments performed to test for effects of adrenal hormones on the resistance of mice to trivalent arsenical compounds and to certain other organic compounds.

Materials and methods. Injections into mice. Compounds under test for lethal toxicity or tumor-damaging potency were injected in aqueous solution either subcutaneously or intravenously. Hormone preparations under test for ability to counteract toxic or tumor-damaging effects of these compounds were usually injected intraperitoneally. Doses for adrenal extract and for 10% alcohol have been stated as cc per mouse or cc per gram. All other substances employed were dissolved or suspended at concentrations such that the desired doses, in mg per kg, were secured by injecting the test solutions or suspensions into mice in the amount of 0.01 cc of solution or suspension per gram of body weight. The amounts of substances injected, the routes of injection, and the strain, sex and numbers of mice used are indicated in the Tables and Figures.

Optimum time for injecting hormone to secure protection. In any one experiment, each mouse in a control group of at least 25 mice received an LD60-100 amount of the toxic compound, but no hormone preparation. Each experimental group consisted of 10 mice, each of which received the selected dose of a hormone preparation, and the same dose of the toxic compound as that administered to mice in the control group. For each experimental group, a different time interval between administration of hormone preparation and administration of toxic compound was employed (Fig. 1). *Lethal dose determinations.* Experiments and calculations were done in accordance with a simplification of Karber's method described by Cornfield and Mantel(3). Generally a given dose of toxic compound was administered to 10 mice, and the doses were varied in 0.1 $\log_{10}$ steps. The time interval between injection of hormone preparation and of toxic compound was in the range indicated by previous experiments as optimal for securing protection of mice against an LD60-100 amount of the toxic compound. *Estimation of gross and histologic damage induced in mouse sarcoma 37 by administration of compounds to mice:* Tumor mash was

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TABLE I. LD Values for Trivalent Arsenical Compounds Injected Subcutaneously into Mice, as Affected by Prior Intraperitoneal Injections of Beef Adrenal Extract.

Exp. No.	Group*	Mice used			LD values (mg/kg)		P values for differences in LD_{50} values of C & E groups
		Strain	Sex	Total No.	LD_{50} ± S.E.	LD_5	
				Experiments with arsenite			
1	C	ZBC	♂	95	12.3 ± .70	8.6	.000004
	Ca	ZBC	♂	95	11.1 ± .37	7.8	
	E	ZBC	♂	95	16.6 ± .62	13.4	
2	C	CAF_1	♂	60	11.2 ± .43	8.6	.00002
	E	CAF_1	♂	60	13.5 ± .33	12.1	
3	C	CAF_1	♀	60	12.5 ± .50	9.3	.04
	E	CAF_1	♀	60	13.7 ± .46	10.3	
4	C	CAF_1	♀	40	10.5 ± .64	6.7	.03
	E	CAF_1	♀	40	12 ± .68	8.2	
				Experiments with clorarsen			
5	C	ABC	♂	80	41 ± 1.8	22.5	.005
	E	ABC	♂	80	48.8 ± 1.5	38.2	
				Experiments with oxophenarsine			
6	C	ABC	♂	30	42.7 ± 2.4	27.1	.03
	E	ABC	♂	30	49 ± 2.2	38.2	

* C or Ca = control, E = exp. C mice received arsenical injections only. Ca mice of Exp. 1 were each given two .5 cc intraperitoneal injections of 10% alcohol, one 40 min before, the other 6 hr after injection of arsenite; E mice of Exp. 1 were each given two .5 cc injections of adrenal extract, one 40 min before, and the other 6 hr after injection of arsenite. In all other experiments, E mice were each injected with .5 cc of adrenal extract 60 min before injection of the trivalent arsenical compound. Mice used in Exp. 2 and 3 bore Sarcoma 37 implanted intramuscularly in the right hind leg 6 days before injection of arsenite.

TABLE II. LD Values for Arsenite Administered to CAF_1 Mice, as Affected by Prior Intraperitoneal Injection of Cortisone.

Exp. No.*	Group	Dose of cortisone (mg/kg)	Time between hormone & arsenite (min)	Mice used		LD values (mg/kg)		P values for differences in LD_{50}s of C & E groups
				Sex	Total No.	LD_{50} ± S.E.	LD_5	
1	C	0	—	♂	24	10 ± .75	—	.01
	E	1.25	50	♂	24	12.6 ± .79	—	
2	C	0	—	♂	50	9.8 ± .59	6.4	.03
	E	2.5	60	♂	50	11.2 ± .41	9	
3	C	0	—	♂	37	12 ± .55	8.6	.01
	Cs	0	—	♂	37	11.7 ± .50	8.9	
	E	2.5	60	♂	37	13.6 ± .35	12.2	
4	C	0	—	♂	70	10.7 ± .42	6.9	.001
	E	2.5	60	♂	60	12.7 ± .38	10	
5	C	0	—	♂	40	10.5 ± .37	9	†
	E	5	90	♂	40	11 ± .46	9.1	
6	C	0	—	♂	20	9.8 ± .55	—	.0001
	E	10	50	♂	20	13.5 ± .62	—	
7	C	0	—	♀	24	10 ± .52	—	†
	E	1.25	50	♀	24	10.4 ± .64	—	
8	C	0	—	♀	50	10 ± .60	6.6	−.02‡
	E	5	90	♀	50	8.5 ± .41	6.5	
9	C	0	—	♀	20	10.3 ± .52	—	†
	E	10	50	♀	20	12.3 ± .69	—	

* In Exp. 4 arsenite was injected intravenously; in all other experiments, subcutaneously. In Exp. 3 an extra control group (Cs) was employed, in which mice were injected intraperitoneally with .9% NaCl, .01 cc per g, 60 min before injection of arsenite. In Exp. 1 and 7 mice bore Sarcoma 37 implanted intramuscularly in right leg 5 days before injection of arsenite.

† Not significant.

‡ Minus value for P indicates augmentation, not diminution, of arsenite toxicity for cortisone injected mice.

TABLE III. Effect of ACTH on LD Values of Arsenite for Normal ABC Mice.

Group	Hormone injected	Mice used		LD values (mg of As_2O_3/kg)		P values for differences in LD_{50} values of C & E groups
		Sex	Total No.	$LD_{50} \pm$ S.E.	LD_5	
C	None	♂	40	11 ± .41	9.1	.01
E	ACTH, 100 mg/kg IP, 24 hr before arsenite	♂	40	12.9 ± .60	10.1	
C	None	♀	40	11.8 ± .42	10.5	*
E	ACTH, 100 mg/kg, IP, 25 hr before arsenite	♀	40	12.6 ± .70	8.9	
C	None	♂	40	11 ± .41	9.1	C vs. E_1: .004
E_1	40 mice received ACTH, 100 mg/kg IP, 24 hr before arsenite. 40 mice received ACTH, 100 mg/kg IP, 24 hr and again 16 hr before arsenite	♂	80	12.6 ± .41	9	
E_2	ACTH, 100 mg/kg IP, at 24 and again at 16 hr before arsenite, + beef adrenal extract 45 min before arsenite	♂	40	16.6 ± .64	14.3	C vs. E_2: .00001

* Not significant.

TABLE IV. Data Obtained in Tests for Protection of Mice by Cortisone Against Semi-Lethal Doses of Compounds Other Than Trivalent Arsenical Compounds, Injected Subcutaneously.

Compound	Dose of cortisone (mg/kg)	Min between cortisone & compound	Mice employed			$LD_{50} \pm$ S.E.*	P values for differences in LD_{50} values for cortisone & control mice
			Strain	Sex	Total No.		
Alpha-peltatin	0	—	CAF_1	♀	40	60.3 ± 4.3	–.002†
	2.5	120	CAF_1	♀	40	43.7 ± 2.7	
"Acridan"‡	0	—	ABC	♂	40	3.63 ± .21	§
	2.5	120	ABC	♂	40	3.55 ± .21	
	0	—	ABC	♀	40	4.17 ± .24	§
	2.5	120	ABC	♀	40	4.34 ± .21	
P-chloromercuribenzoate	0	—	CAF_1	♂	40	75.9 ± 2.7	§
	5	60	CAF_1	♂	40	79.5 ± 3.1	
Iodoacetamide	0	—	CAF_1	♂	65	42 ± 1	§
	5	60	CAF_1	♂	65	40.8 ± 1.8	

* LD_{50} values are for 7 days after injection of alpha-peltatin; for 48 hr after injection of other compounds.

† Minus probability value indicates *augmentation* of toxicity by cortisone.

‡ 9-phenyl-9-chloro-10-methyl-3-dimethyl amino acridan 2 HCl.

§ Not significant.

implanted in the right hind leg of CAF_1 mice. Six or 7 days later injections of the potentially tumor-damaging compound, and of hormone preparations, were made. The mice were sacrificed 24 hours after injection of the compound under test for tumor-damaging potency and the extent of induced hemorrhagic necrosis was estimated grossly by visual inspections of cross sections of the tumors *in situ* (5 mice per group). Sections were obtained and histological estimations of extent of induced damage were made by criteria described by MacCardle and Downing(4), and by Leiter, Downing, Hartwell and Shear(5). In this paper, attention has been focused on *comparison,* at each dose level of tumor-damaging

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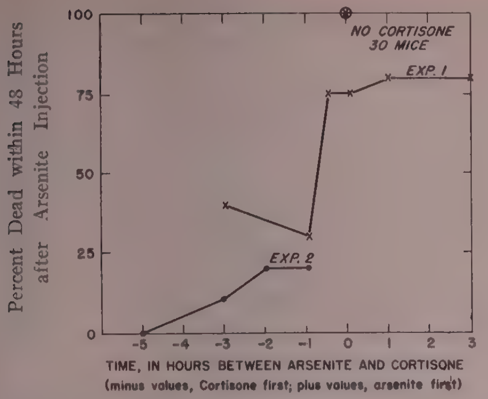

Mice: ABC males, 10 per point
Arsenite: 12.6 mg. of As2O3 per kg., subcutaneously
Cortisone: 2.5 mg. per kg. intraperitoneally

FIG. 1. Relation between time of administration of cortisone and protection afforded mice against arsenite.

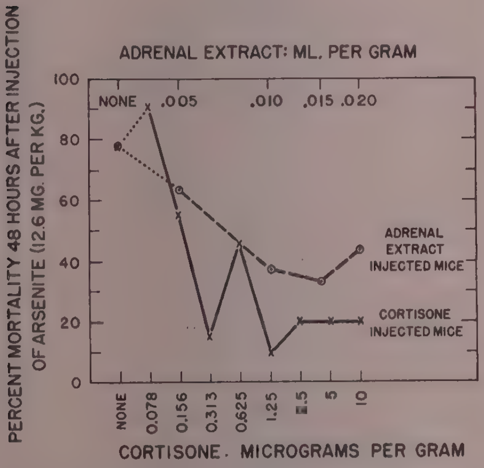

FIG. 2. Protection afforded mice against arsenite by adrenal hormones, in relation to dose of hormone administered.

compound, of the amount of induced damage found in tumors from mice which (a) *did*, and (b) *did not* receive a prior injection of a hormone preparation.

Compounds under test for lethal toxicity and tumor-damaging potency. Arsenite. Fowler's solution, pH 9.6, was prepared as described in the Pharmacopoeia of the United States, using arsenious acid, C. P., Baker's Analyzed. Any unused portion of this solution was discarded within 4 weeks after preparation. Solutions of arsenite at the desired concentrations for injections into mice were secured by diluting Fowler's solution with water. All doses for arsenite or arsenious acid stated here refer to dry arsenous acid. Arsenite, having certain advantages over various aromatic trivalent arsenical compounds, was used for most of the experiments. It is more stable, both in solid form and in solution than most, if not all, organic trivalent arsenical compounds. The difference in arsenite LD0 and LD100 is relatively small, so that significant changes in arsenite LD50 induced by prior treatment with hormone preparations could be determined without the use of excessively large numbers of mice. The minimum dose of arsenite required to induce marked gross and histological damage to mouse Sarcoma 37 did not vary appreciably from experiment to experiment. *Oxophenarsine.* Preparations of 3-amino-4-hydroxyphenylarsenoxide HCl as a dry powder in sealed ampoules[†] were used. Just before preparation of a solution of oxophenarsine, it was mixed in the dry form with 4.3 parts by weight of a dry, powdery proprietary citrate-acetate buffer mixture.[‡] Solutions of the mixture (pH values about 6.5) were injected into mice as soon as possible after preparation to minimize chemical change in solution. All doses for oxophenarsine refer to the dry form, before mixture with the buffer mixture. *Clorarsen.* Powder preparations in sealed ampoules of 3-amino-4-hydroxyphenyldichlorarsine HCl, mixed with 3.3 parts by weight of a proprietary citrate-acetate buffer, were used.[‡] Solutions had pH values about 5.6. Stated doses are for the pure compound. *P-chloromercuribenzoic acid.* In the form of dry powder[§] it was dissolved in water plus an equimolecular amount of NaOH. *Iodoacetamide* was secured as powder.[¶] *Alpha-peltatin* was secured from the Organic Chemistry Unit, Chemotherapy Section, National Cancer Institute. *"Acridan."* 9-phenyl-9-chloro-10-methyl-3-dimethyl amino acridan 2 HCl was secured as a powder from the Malaria Control Board, National Institutes of Health, Bethesda, Md.

[†] Parke-Davis and Co., Mapharsen.
[‡] Squibb and Sons, Inc.
[§] Donated by Dr. Leslie Hellerman
[¶] Eastman Kodak Co.

Hormone preparations tested for ability to protect mice or mouse sarcoma 37 against toxic and tumor-damaging compounds. Beef adrenal extract‖ obtained in 10 cc sealed ampoules, prepared commercially was generally administered in the amount of 0.5 cc per mouse. In some experiments, control mice were injected with 0.5 cc of 10% alcohol, since the alcohol content of the beef adrenal extract was 10%. *Adrenocorticotropic hormone (ACTH)*** had a stated assay value of 57% of the activity of Armour and Co. standard ACTH preparation. All doses stated here are for the preparation used, uncorrected for difference in assay from this standard. The compound was injected in water solution. *Cortisone.*†† A suspension of 11-dehydro-17-hydroxycorticosterone-21- acetate (25 mg per cc) in 0.9% NaCl was used. Suspensions for injections into mice were prepared by diluting the stock suspensions with 0.9% NaCl, just prior to an experiment. *Desoxycorticosterone acetate.*†† A suspension of the compound (25 mg per cc) in 0.9% NaCl was used. Suspensions for injections into mice were prepared as stated above for cortisone.

Results. I. Experiments with trivalent arsenical compounds. Optimum time interval between hormone and arsenite. Results obtained with ABC mice and cortisone, shown in Fig. 1, indicate that protection against arsenite could be secured by injecting cortisone over a wide time interval prior to injection of arsenite. For the beef adrenal extract, maximum protection against arsenite was secured by injection of extract about an hour before injection of arsenite. In similar experiments in which 2.5 or 5 mg of desoxycorticosterone acetate per kg was used and the time interval between hormone and arsenite was varied from zero to 24 hours, no evidence of protection was secured. Measurable protection was obtained by administration of ACTH 5 to 24 hours prior to injection of arsenite. Measurable protection was not obtained by injecting any of these substances *after* injection of arsenite. *Minimum dose of hormone required to secure protection against arsenite.* Data obtained in tests using ABC male mice are shown in Fig. 2. Hormone doses are plotted on a logarithmic scale. The time interval between injection of cortisone and injection of arsenite was 50 minutes, that between injection of adrenal extract and injection of arsenite, 60 minutes. The data indicate that for mice subsequently injected with 12.6 mg of arsenite per kg, maximum protection obtainable with the extract was obtained by the use of as little as 0.01 cc of extract per gram of body weight, and that considerable protection was secured with as little as 0.31 mg of cortisone per kg. This corresponds to less than one molecule of cortisone for every hundred molecules of arsenite administered. *Lethal dose values obtained for arsenite, oxophenarsine and clorarsen, in male and female mice, as affected by hormones.* Data obtained in experiments in which the beef adrenal extract was injected 40 to 60 minutes prior to the arsenical compound are shown in Table I. This extract afforded male mice significant protection against arsenite, against oxophenarsine and against clorarsen. This protection was not due to the water-alcohol content of the adrenal extract (Exp. 1, Table I). Much less protection was afforded female than male mice against arsenite. From data obtained in experiments using cortisone as the test protecting hormone, and shown in Table II, it is seen that significant protection was afforded male mice against arsenite, injected either subcutaneously or intravenously, by cortisone injected intraperitoneally. In experiments for which data are not included, male mice were found to be protected against arsenite injected subcutaneously, by cortisone injected subcutaneously at another site. Protection by cortisone was not due to the water-NaCl content of the cortisone suspension (Exp. 3, Table II). Cortisone did not afford female mice significant protection against arsenite in any one of the 3 experiments performed. In 8 experiments with male mice in which both LD50 and LD5 values were calculated (Tables I and II), the average percent increase in LD50 value obtained by pretreatment with adrenal extract or cortisone was 18%, whereas the average increase in LD5 value was 42%. Table III gives data obtained in experiments using ACTH and arsenite. In 2 experiments in which male mice were employed a statistically significant protection was noted. In an experiment in which male mice were injected with both ACTH and beef adrenal extract, quite marked protection was obtained against arsenite. In one experiment with female mice, no protection was noted. *Tumor-damaging effects of arsenite, as affected by adrenal hormone preparations.* In an experiment with CAF_1 males and in another with CAF_1 females, the minimum effective dose of arsenite for inducing marked hemorrhagic necrosis in Sarcoma 37 was judged to be 10 mg per kg for control mice, and 12.6 mg per kg for mice which had been injected with the beef adrenal extract, 0.5 cc per mouse, one hour prior to injection of arsenite. In an experiment with CAF_1 males, the minimum effective dose of arsenite for inducing marked hemorrhagic necrosis in Sarcoma 37 was judged to be 8 mg per kg for control mice, and 10 mg per kg for mice which had been injected with cortisone in the amount of 1.25 mg per kg, one hour prior to injection of arsenite. Since (a) the minimum dose of arsenite effective in inducing marked necrosis in Sarcoma 37, and (b) arsenite MTD for mice (LD5 of Tables) were both increased by prior administration of adrenal extract or cortisone, it would appear that in mice prior administration of these hormone preparations was not markedly effective in increasing the margin of safety in the use of arsenite as a tumor damaging agent.

‖ Upjohn Pharmaceutical Co.

** Armour and Co.

†† Merck and Co.

II. *Experiments with non-arsenical organic compounds.* Table IV shows lethal dose values obtained for a number of tumor-damaging compounds. Under the conditions employed, there was no evidence of protection of mice by cortisone against semi-lethal amounts of any of these compounds. The negative findings with p-chloromercuribenzoic acid and iodoacetamide are of particular interest, since they are classified with trivalent arsenical compounds as sulfhydryl poisons(6).

Discussion. The present experiments do not provide a basis for stating the mechanism whereby measurable protection against semi-lethal amounts of arsenite, oxophenarsine and clorarsen was conferred on male mice by certain of the adrenal hormones. Experiments are in progress in this laboratory to test for effects of adrenal hormones on tissue concentrations of sulfhydryl compounds capable of binding and so detoxifying trivalent arsenical compounds. Adrenal hormone preparations have generally failed to increase the resistance of normal (non-adrenalectomized) animals to toxic substances. However, Clark and Barnes (7), using a few animals, have reported protection of rats against colchicine by cortin. Zwemer and Jungeblut(8) have reported some protection of guinea pigs against diphtheria toxin by adrenal extract. Ingle(9) has reported protection of rats against peptone shock by adrenal extracts. Beck and Voloshin (10) have reported protection of mice against *Serratia marcescens* tumor-necrotizing polysaccharide by cortisone. Vollmer (unpublished) has found that mice may be protected against semi-lethal doses of potassium, not only by certain adrenal hormones, but also like arsenite by glutathione.

Summary. Prior administration of beef adrenal extract increased the resistance of mice, particularly male mice, to semi-lethal doses of arsenite, oxophenarsine and clorarsen. Male mice were protected against arsenite by cortisone and ACTH, but not by desoxycorticosterone. Prior administration of cortisone did not increase the resistance of mice to semi-lethal doses of p-chloromercuribenzoate, iodoacetamide, alphapeltatin, or 9-phenyl-9-chlor-10-methyl-3-dimethyl amino acridan. The minimum dose of arsenite required to induce marked hemorrhagic necrosis in mouse Sarcoma 37 was somewhat greater for adrenal extract or cortisone injected mice than for control mice.

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Abnormalities in Offspring of White Rats Given Protamin Zinc Insulin During Pregnancy.* (19084)

Henrik Lichtenstein,† George M. Guest, and Josef Warkany.

From the Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati.

The studies reported here were designed to determine what effects the injection of large doses of protamine insulin in pregnant rats might have on fetal development. They were undertaken as an extension of investigations on the influence of various environmental factors on the occurrence of congenital malformations in the rat that have been described in various reports from this laboratory (1-3).

Interest in the possibility that insulin injected into pregnant animals might have teratogenic effects on fetal development has been stimulated by reports that insulin injected into developing eggs produced malformations in chick embryos. Landauer(4-6) found that insulin injected into the yolk sac led to rumplessness and abnormalities of the beak, extremities and eyes; others have confirmed these findings(7,8). Recently Duraiswami(9) reported the production of gross skeletal abnormalities in developing chicks by injections of crystalline insulin into developing eggs on the first to the sixth days of incubation. Duraiswami found that the growth of the embryo as a whole was retarded to a degree varying with the dose of insulin, and that different parts of the body were affected to varying degrees according to different days when the injections were done. Primary effects on different parts of the body apparently depended on which part or organ was in the most active stage of development at the time of the injection. Ferrill reported (10) that prolonged administration of subshock doses of insulin in rats, 20 to 40 units per kg of body weight per day (presumably unmodified insulin) had no significant effects on reproduction in the first generation or the succeeding 5 generations. The report does not mention malformations.

Methods. The studies were done during the period from March, 1949, to May, 1950, using young albino rats that were reared on a diet known to be adequate for growth and development, rich in B-complex. The females were not mated until they attained a weight of 150 g. Kept in individual cages, they were weighed and vaginal smears were examined daily; when they were found to be in oestrus, males were placed with them. The day on which sperm were found in the vaginal smear was recorded as the first day of pregnancy, with recognition that this tabulation involved possible errors up to 24 hours in calculating gestation periods. Trials of various dosages of protamin zinc insulin (PZI) were made to determine the maximum amount that would permit survival and good reproduction. Six pregnant rats given 10 units of PZI daily died within 6 days, although non-pregnant rats tolerated considerably higher doses for longer periods. Seven to 8 units of PZI given subcutaneously each day as a single dose appeared to be the maximum amount tolerated.‡ With this dosage, adopted for the experiments, there was a wide variation in mortality rates, ranging from 15 to 75% among different

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* Aided by a grant from the Nutrition Foundation, New York City.

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‡ The insulin was the U-40 solution, supplied by Eli Lilly Co., Indianapolis.

groups and at different times. The highest mortality occurred during the very warm summer of 1949. The insulin was injected daily throughout the gestation period, beginning on the second day of pregnancy or earlier. In a few instances when vaginal bleeding, maternal loss of weight or failure to gain indicated that the fetuses were probably resorbed, the insulin injections were not interrupted but the rat was remated and the injections then continued during a new pregnancy. Injections of insulin before mating did not notably affect the capacity for conception or the course of gestation. A control group of 41 rats received no injections of any sort; but in another investigation conducted at about the same time in the same laboratory, a control series of normal pregnant rats received injections of 0.2 cc of 0.9% solution of NaCl subcutaneously daily or several times a day without apparent effect on gestation. Of 55 rats injected with insulin, 24 received 7 units and 31 received 8 units daily.

The 96 rats included in the study produced (in 96 litters) 783 fetuses. Some rats were allowed to await normal delivery, expected normally on the 23rd day of pregnancy, but most of them were anesthetized and opened on the 22nd day. Before the 22nd day a few rats delivered spontaneously or were opened when signs of probable resorption appeared. A few rats found dead on the 21st day were opened and the dead fetuses were removed and included in the series. None of the latter fetuses showed signs of maceration nor external signs of developmental abnormalities. The fetuses in each litter were counted and in most cases weighed, and then by random selection they were either placed in alcohol for clearing and staining bone by the Schultze-Dawson method(11), or placed in formalin to be fixed for later serial sectioning. The control group of 41 litters included 371 fetuses, of which 176, or 47.4%, were cleared and stained by the Schultze-Dawson method. Fifty-five litters from insulin-treated rats included 417 fetuses, of which 257, or 62.4%, were similarly prepared. The present report concerns only studies of skeletal development; detailed studies of the fetal soft tissues have not yet been done. Comparisons between skeletal development in the control and insulin-treated groups of fetuses are limited to litters counted as having had 22 days of gestation.

Results. Among the rats that delivered their young spontaneously no differences in length of gestation (21 to 23 days) could be discerned between the control and insulin-treated groups. The majority of the rats in both groups were, however, killed on the 22nd day of pregnancy for removal of the fetuses. The average weight of the fetuses with 22-day gestation periods was 5 g in the control group, 4.1 g in the insulin-treated groups.

The control series of 41 rats not treated with insulin yielded 371 fetuses, an average of 9 per litter. The 55 rats treated with insulin yielded 417 fetuses, an average of 7.6 per litter. Counts of resorption sites in the uteri accounted for the differences in litter size. Among 24 rats that received 7 units of insulin per day, 24 resorption sites were counted in 9 (15 showing none). Among 31 rats that received 8 units daily, 87 resorption sites were counted in 24 (only 7 showing none). The total number of 117 resorption sites added to the total number of fetuses in these two groups would bring the number of conceptions to 528, or 9.9 per litter. Among the control rats resorption sites were rarely seen.

On superficial examination all the fetuses from both control and insulin-treated rats appeared normal, with one exception. This one was in a litter of 13, with average weight 3.7 g, which were removed alive from a rat that received 7 units of insulin daily. This fetus was edematous and appeared to have short legs, with poor differentiation of the fingers and toes, and a crooked tail; it was fixed in formalin and serial sections made for histologic studies to be reported later. Among fetuses cleared and stained for skeletal visualization, no noteworthy abnormalities were found in the control group, but a number of abnormalities appeared among fetuses of the insulin-treated group. Photographs in Fig. 1

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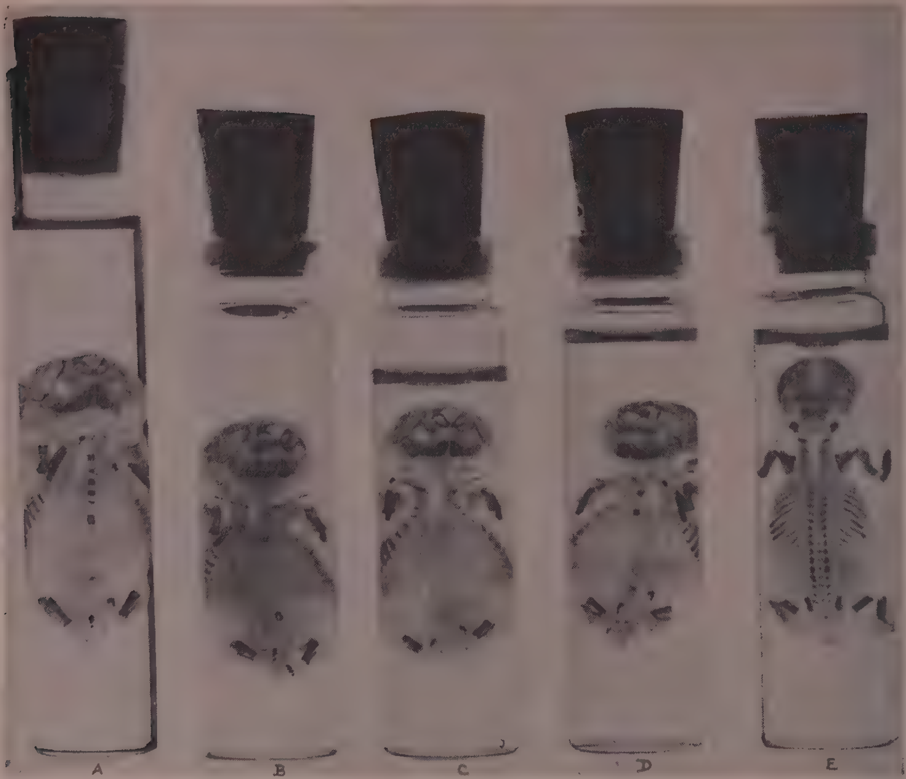

FIG. 1. Fetuses cleared and stained by the Schultze-Dawson method: one (A) from a normal control rat and 4 (B, C, D, E) from rats that received 8 units of protamine zinc insulin daily, all with gestation periods 22 days. A. Normal sternum with 6 well stained sternebrae. B. One well stained sternebra. C. No stained sternebrae. D. Two well stained sternebrae. E. Rear view, showing "wavy" ribs.

and 2 illustrate the absence of visible sternebrae and abnormalities of ossification in the ribs and long bones of the extremities in a few fetuses selected as examples. Among fetuses of rats that received 7 units of insulin daily, "wavy" ribs were seen in 2 fetuses in each of 3 litters, without other visible abnormalities of ossification. Among 4 litters of rats that received 8 units daily, wavy ribs were seen in 1 fetus of each of 2 litters, and in 3 fetuses of each of 2 other litters. In some of the cleared fetuses of the insulin-treated rats the femur and humerus seemed short in relation to body size; occasionally, irregularities were visible in the ends of the femur and tibia, suggesting irregular ossification.

In Table I are listed the numbers of sternebrae seen among cleared and stained fetuses of the control and insulin-treated groups. In the control group of 28 litters with a gestation period of 22 days, 83 cleared fetuses showed 6 well stained sternebrae and 1 showed 5. The latter was in a litter in which the average fetal weight was only 3.6 g. Among 53 cleared fetuses from 11 litters of rats that received 7 units of insulin daily, 50 showed 6 visible sternebrae (some of them poorly stained); of the remaining 3, two showed 3 stained sternebrae and one none. Among 129 cleared fetuses from rats that received 8 units of insulin daily, 66 had 6 sternebrae (visible though many were poorly stained); among the remaining 63 fetuses the visible sternebrae ranged in number from 5 to 0.

Discussion. Strong(12) states that the

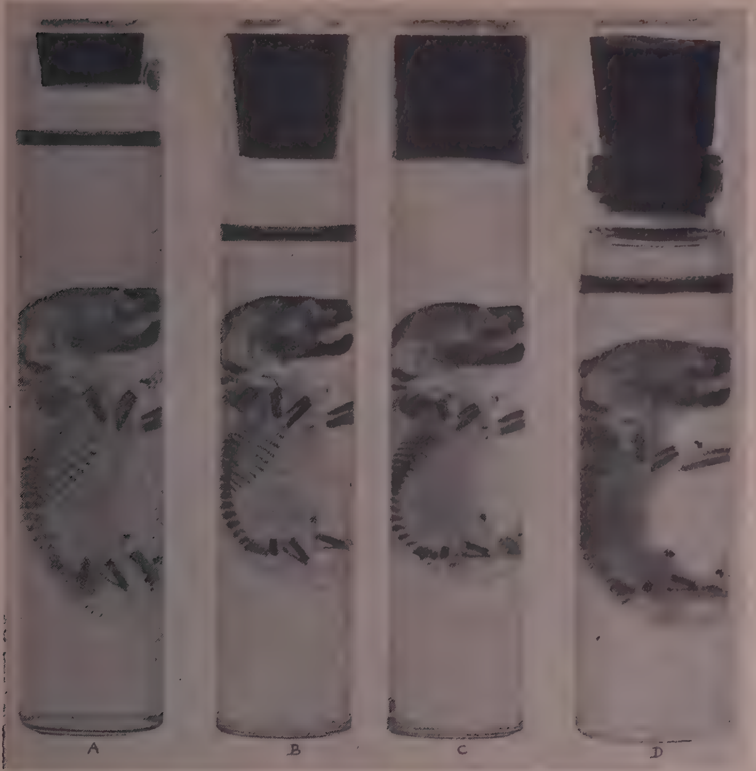

FIG. 2. One fetus from a normal control rat and 3 from rats that received 8 units of protamine zinc insulin daily, all with gestation periods 22 days. A. Normal sternum with 6 well stained sternebrae, normal ribs and normal long bones. B. One well stained and 2 poorly stained sternebrae. C. Three well stained and 3 poorly stained sternebrae, irregular formation of the radius and ulna. The right humerus and the femura are short. The tibiae end obliquely and their staining is irregular. ''Wavy'' ribs. D. No sternebrae are stained. The femurae are short. The tibia ends are oblique and irregular.

sternum of the albino rat consists of 6 sternebrae. After 19 days, 9¾ hours of gestation ossification centers are already present in all except one or two, and at 21 days all 6 sternebrae are ossified. This was essentially the condition found in our control litters with 22-day gestation period.

In the fetuses from insulin-treated rats the decreased number and/or lack of ossification centers in the sternum, and differences in depth of staining, suggests abnormalities in ossification of the centers, possibly other developmental abnormalities in cellular structure. There appeared to be a parallel comparable retardation of development of the carpals, metacarpals, the phalanges and the vertebrae of the tail.

Since the average weight of fetuses from the insulin-treated rats was less than that of normal controls, it seems possible that the insulin slowed fetal development, directly or indirectly. It is uncertain whether insulin passes through the placenta of the rat(13), and what role changing levels of sugar in maternal and fetal blood may play.

Allen suggested(14) that insulin, especially protamin zinc insulin, may have toxic effects other than those ascribable to hypoglycemia;

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TABLE I. No. of Ossified Sternebrae in Fetuses of Control and Insulin-Treated Rats with 22 Days Gestation.

	28 litters Control		11 litters Insulin, 7 units/day		29 litters Insulin, 8 units/day		
Stained sternebrae	No. of fetuses	%	No. of fetuses	%	No. of fetuses	%	
6	83	98.8	50	94.3	66	51.16	66.66
5	1	1.2	0	0	10	7.75	
4	0	0	0	0	10	7.75	
3	0	0	2	3.8	13	10.08	33.34
2	0	0	0	0	11	8.53	
1	0	0	0	0	10	7.75	
0	0	0	1	1.9	9	6.98	
Total	84		53		129		

this possibility must of course be considered in the interpretation of results such as those reported here. Duraiswami(9) found, however, that inactivation of insulin by the methyl-alcohol-hydrochloric acid method of Charles and Scott rendered it ineffective in producing abnormalities in developing chicks; in his experiments the effect of insulin on developing chick embryos appeared to be linked with its blood-sugar lowering action. After noting that the skeletal abnormalities induced by insulin in chicks resembled the abnormalities produced in rats by maternal diets deficient in riboflavin, as reported by Warkany and coworkers(1-3), Duraiswami speculated on the possibility that insulin induced hypoglycemia might involve disturbances in phosphorylating enzyme systems such as arise with a lack of vit. B complex, thus causing disturbances of carbohydrate metabolism in embryonic tissue during critical periods of development. Giroud's studies(15,16) on the effects of avitaminosis-B_2 on embryonic development in the rat also are pertinent to such speculations.

Summary. Protamine zinc insulin administered to 51 pregnant albino rats throughout pregnancy in doses 7 and 8 units daily (nearly the maximum dose tolerated) had significant effects on fetal development. Compared with fetuses of untreated normal control rats, the average weight of the fetuses of the insulin-treated rats was lower, and the number in each litter was less owing to fetal deaths and resorption. Mild skeletal abnormalities were found, many of the fetuses showing a decreased number or complete absence of ossification centers in the sternum, a few showing some irregularities in shape and staining of the ribs and long bones.

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Effect of Protein Depletion on Rate of Disappearance of Radioactive Protein from the Blood Stream. (19085)

CHARLES A. HUBAY, HARVEY KRIEGER, WILLIAM D. HOLDEN, AND THOMAS HANCOCK.

From the Department of Surgical Research, Western Reserve University School of Medicine and the Department of Surgery, University Hospitals of Cleveland.

The relationship of the effective circulating blood volume and disease states has assumed an important place in surgical physiology. Several methods of determining the blood volume have been advanced, including the use of radioactive (I-131) iodinated plasma protein. Storaasli(1) and his co-workers demonstrated that the stability of the iodinated protein and its relatively secure retention within the vascular system permits a more accurate estimate of the plasma volume than has hitherto been possible. Previous work (1-5) has shown that after radioactive iodinated plasma protein is injected intravenously, there is a progressive decrease in the measurable radioactivity of blood samples withdrawn periodically. Storaasli, *et al.*(1) showed that the method of disappearance of iodinated protein from the vascular system is two fold: (a) by the diffusion of the protein into extra vascular spaces shown by studies of thoracic duct lymph(3,4) and (b) by liberation of the radioactive iodine from the protein as manifested by excretion of iodine in the urine and increased radioactivity over the thyroid gland.

This method of blood volume determination was shown to be reasonably accurate in normal individuals and we studied its use in pathophysiological states. The work reported here was done in experimental animals and human patients with hypoproteinemia. The existence of endogenous reserve stores of protein has been demonstrated by Whipple(6). When these reserve stores are depleted by inadequate diet, excessive loss, or metabolic dysfunction, the ability of tissues to withstand trauma and to repair themselves is decreased. It was thought worthwhile to investigate the rate of disappearance of tagged protein from the blood stream to see whether or not the state of the protein reserves of the body could be determined by deviations from the normal disappearance rate. This has great clinical importance, particularly in surgery, in determining the optimum time for operation in malnourished and debilitated patients.

Method. The disappearance rates (Fig. 1) of tagged protein were determined in normal healthy mongrel dogs, on standard kennel diets. These dogs were then placed on a low protein diet, similar to the one employed in G. H. Whipple's laboratory(7). Nitrogen balance studies were not feasible but the amount of nitrogen per day in the diet did not exceed one gram. Eleven dogs were studied but several animals refused to eat this low protein diet and depletion studies were possible in only 5 animals. After 4 to 8 weeks on this low protein diet disappearance rates of radioactive protein were again determined. During this time the plasma protein levels and body weights were determined weekly. Following these periods of protein deprivation, an attempt was made to replete the animals to a normal nutritional state by placing them on the standard kennel diet, but all died of intercurrent infection or distemper, except animal No. PD 5 which recovered. The radioactive (I-131) protein* was prepared from dog plasma by a modification of the method of Fine and Seligman(8). Preparations are now available utilizing human serum albumin for study of human patients.† The technic used in determining the plasma volume involves the measurement of the dilution of tagged protein after intravenous injection and has been described(1,2). Twenty cubic centimeters of the tagged protein solution were injected intravenously and periodic venous samples were withdrawn without stasis at 10, 15, 30 minutes, 1, 2, 4, 6, 8, 12 and 24 hour periods. The blood was placed in tubes containing crystalline heparin and then centrifuged at 2500 RPM for 30 minutes. The

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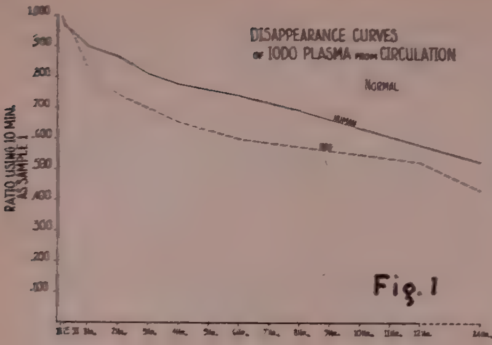
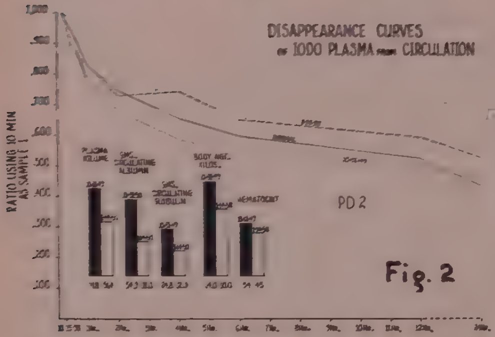
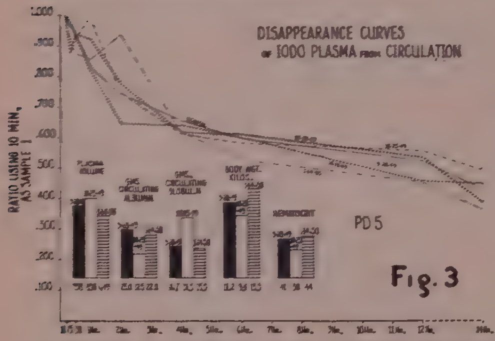
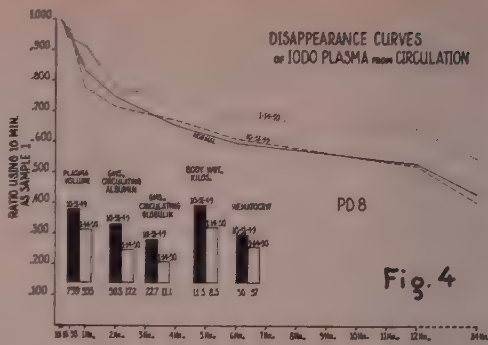
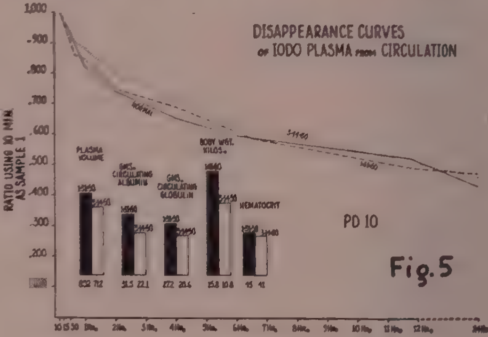
† The Abbott Laboratories generously supplied us with the radioactive albumin used in human cases in this study.











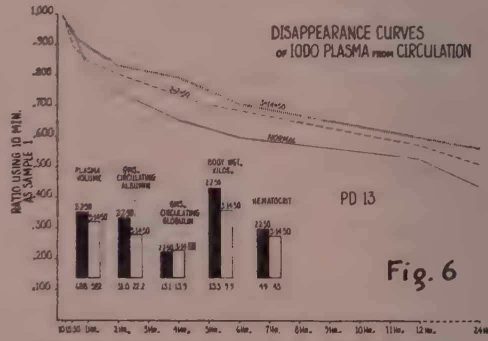


FIG. 1. Average rates of disappearance of radioactive plasma protein in normal human patients and dogs. Reproduced in part from Storaasli *et al.*, *Surg. Gyn. & Obst.*, Oct., 1950, by permission of the publishers.

FIG. 2. Disappearance curves of radioactive plasma protein from circulation of dog PD 2; basal condition 10-31-49, protein depletion, 1-14-50.

FIG. 3. Disappearance curves of radioactive plasma protein from circulation of dog PD 5; basal condition 9-28-49; protein depletion 10-25-49; protein repletion 1-14-50.

FIG. 4. Disappearance curves of radioactive plasma protein from circulation of dog PD 8; basal condition 10-31-49; protein depletion 1-14-50.

FIG. 5. Disappearance curves of radioactive plasma protein from circulation of dog PD 10; basal condition 2-2-50; protein depletion 3-14-50.

FIG. 6. Disappearance curves of radioactive plasma protein from circulation of dog PD 13; basal condition 2-2-50; protein depletion 3-14-50.

supernatant plasma was removed and 1 cc samples were then counted by means of a Geiger-Muller tube and the activity determined. Total protein, A/G ratios were determined by the Kingsley(9) method utiliz-

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9. Kingsley, G. R., *J. Lab. and Clin. Med.*, 1942, v27, 840.

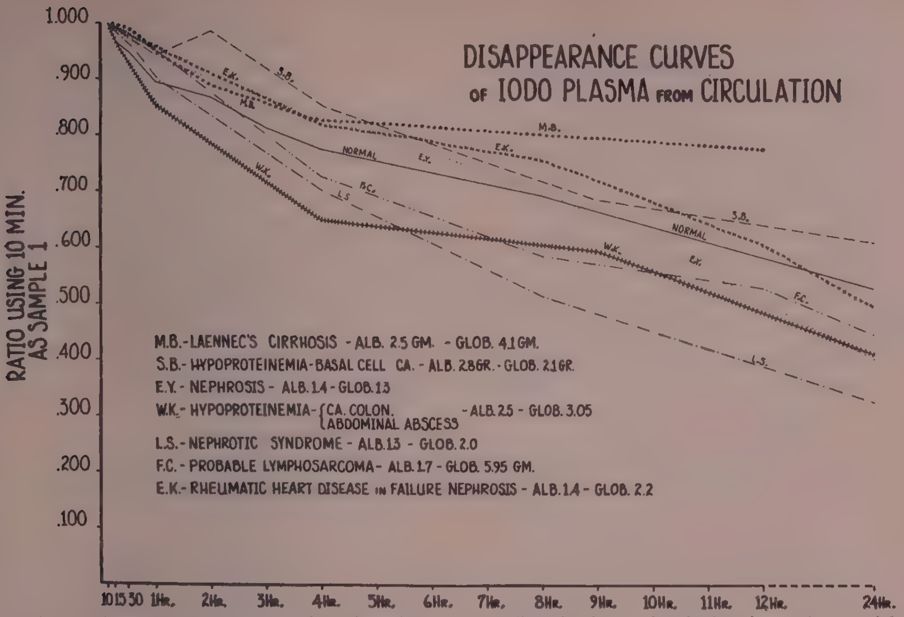

FIG. 7. Disappearance curves of radioactive human albumin from circulation in patients with hypoproteinemia. Expected normal rate of disappearance is shown.

ing the Klett-Summerson colorimeter. Disappearance rates were calculated as activity per cc of plasma and expressed as a ratio using the 10-minute sample as 1.000.

Results. The average normal rate of disappearance of radioactive plasma protein in 11 normal dogs is shown in Fig. 1. This rate of disappearance correlates closely with that described by Fine and Seligman(8) and Cope and Moore(4). Fig. 2 to 6 represent the graphic rates of disappearance of each dog studied with weight loss and hypoproteinemia. In general the differences tend to be an average of 6% slower in the depleted state, and we have shown that at least in one dog (PD 5) this decreased rate of disappearance in the hypoproteinemic state was reversed when the dog was repleted to a normal nutritional state.

On the basis of this study, we do not believe that this diminution in rate of disappearance in the hypoproteinemic state is significant. We feel justified in concluding that the rate of disappearance of radioactive plasma protein cannot be utilized in estimating the state of the protein reserves of the tissues in the dog. Metcalf(10) studied dogs placed on protein free diets and then plasmapheresed them to near edema levels. He found that transfused serum disappeared in protein depleted animals in quantities of the same magnitude as in control animals, and that after protein repletion, the disappearance curves were essentially unchanged.

The plasma volumes and disappearance rates in normal individuals were studied in 30 patients by Storaasli(1). The average disappearance curve is shown in Fig. 1. Seven patients with hypoproteinemia were studied and disappearance curves calculated after injection with tagged (I-131) human serum albumin (Fig. 7). In these patients the rate of disappearance of radioactive iodinated albumin parallels the average normal curve, and the differences seen are probably due to variation in fluid intake, ambulation, etc. In the patients who are losing abnormal amounts of protein in the urine (E. Y., L. S., E. K.) or into abscess cavities (W. K.), the rate of disappearance appears to be increased. In the patients with nephrosis, there is an in-

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10. Metcalf, Wm., *J. Clin. Invest.*, 1944, v23, 403.

creased amount of radioactive iodine recoverable from the urine in a 24-hour period (38%) over that found in the urine of the normal patient (8-12%).

Summary. 1. Plasma volumes can be accurately determined by the radioactive (I-131) iodinated plasma protein method in nutritional hypoproteinemia. 2. The rate of disappearance of radioactive protein from the blood stream is the same in undernourished states as in normal and therefore cannot be utilized to determine the state of the protein reserves of the body. (3). In patients who are losing abnormal amounts of protein in the urine or into abscess cavities, the rate of disappearance appears to be increased. The use of radioactive serum albumin may offer a diagnostic aid in the detection of unexplained protein losses.

The authors gratefully acknowledge the technical assistance of Miss Jean Hower and Mrs. Eileen Davis and wish to thank Doctors Ole Jonassen and Frank Cebul for their aid in the completion of some of these studies.

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In vivo Staining of Fat in Tumor Bearing Mice by Benzo[a]phenoxazine Dyes.* (19086)

MARGARET REED LEWIS, M. L. CROSSLEY, AND P. F. DREISBACH.

From the Wistar Institute of Anatomy and Biology, Philadelphia, Pa., and the Calco Chemical Division, American Cyanamid Co., Bound Brook, N. J.

In a previous study(1) of the effects of the oral administration of dyes on tumor bearing mice it was found that certain benzo[a]phenoxazines containing 5-phenylamino groups stained fat *in vivo*. The N-phenyl derivative of Nile Blue A stained fat a brilliant orange and tumor tissue a pale blue color. On the other hand, it was shown that the 5-amino and 5-benzylamino - 9 - diethylaminobenzophenoxazines, Nile Blue A and Nile Blue 2B, respectively, stained tumor tissue deep blue but failed to stain fat *in vivo*. The failure of the 5-amino and the 5-benzylamino-9-diethylaminobenzophenoxazines to stain fat *in vivo* was of interest because Thorpe(2), Smith(3), and Heidenhain(4) had shown that these compounds stain fat *in vitro*. The *in vitro* differential fat staining effect was supposed to depend to some extent upon the fat solubility of the dyes or their chemical transformation products. Hadjioloff(5), however, in his review of the publications on *in vivo* staining of fat administered by the enteric route, showed that many fat soluble dyes failed to stain fat *in vivo*. In another study of the benzo[a]phenoxazine dyes(6) it was found that of the dyes available at that time only those containing the 5-phenylamino group stained fat *in vivo*.

The present study is concerned with *in vivo* staining of fat in tumor bearing mice following oral administration of a number of substituted 5,9-diaminobenzo[a]phenoxazine dyes. The majority of these contained a 5-phenylamino group while the others had benzyl-, naphthyl- or heterocyclic amino groups in the 5-position. Generally, the hydrogens of the 9-amino group were replaced by alkyl radicals containing from one to 6 carbon atoms.

Material and method. Mice of inbred stains and tumors which were 100% transplantable in mice of the strain of the host were utilized in these studies. A few tumor bearing rats were also used for a comparison of the staining behavior of animals of a different species. The dyes to be tested were ground together with pulverized Purina fox chow in concentrations of 0.2% with the exception of 5-benzylamino-9-dibutylamino, 5-(4-chlorobenzylamino)-9-dibutylamino and 5-(4-methylbenzylamino) - 9-dibutylamino-benzo[a]phenoxazonium chlorides, which were prepared in 0.05% concentrations. The mixtures were fed to the animals for 15 days, beginning on the day the tumor tissue was implanted. At the end of this period, the animals were sacrificed and the results observed. Generally the dyes were used in the form of their salts, such as their chlorides or nitrates, although in a number of instances the dye bases were administered. The preparation of these compounds has recently been described(7).

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* Aided in part by a grant to Dr. Margaret Reed Lewis from the National Cancer Institute.

1. Lewis, M. R., Goland, P. P., and Sloviter, H. A., *Anat. Rec.*, 1946, v96, 201.
2. Thorpe, J. F., *J. Chem. Soc.*, 1907, v91, 324.
3. Smith, J. L., *J. Path. and Bact.*, 1908, v12, 1.
4. Heidenhain, Martin, *Arch. ges. Physiol.*, 1902, v90, 115.
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Results. In these investigations of the effect of benzo[a]phenoxazine dyes on fats and tumors in mice, it was found that the majority of the 5-phenylaminobenzo[a]phenoxazine compounds used stained fat *in vivo* (Table IA). The exceptions were those containing hydroxyl and carboxyl groups on the phenyl nucleus. All of the dyes studied were 5,9-diamino derivatives. In most instances the substituents on the 9-amino nitrogen were alkyl groups, but in certain compounds the carbon chains were bridged by an oxygen atom, giving the morpholinyl radical. This ring structure in the 9-position did not change the behavior of the dyes in the *in vivo* staining of fats.

The phenyl radical substituted for one hydrogen atom of the 5-amino group decreased the *in vivo* tumor staining properties of the resulting dyes, while the benzyl radical increased these properties.

5-benzylamino-9-dimethylamino- and 5-benzylamino- 9 -diethylaminobenzo[a]phenoxazine dyes or their benzyl ring substitution products stained tumor tissue, but all failed to stain fat *in vivo*. They are, therefore, not included in the tables. As is shown in Table 1B, however, the corresponding 5-benzylamino compounds containing alkyl groups of more than 2 carbon atoms each on the 9-nitrogen did stain fat *in vivo*. While all of the dyes with propyl or butyl substituents in the 9-position stained fat, not all of them stained tumor tissue *in vivo*.

As was shown in earlier studies(1,6), the results obtained with Nile Blue staining of tumors in rats were inconsistent and unpredictable. Tumors became stained in only a few rats fed Nile Blue A or Nile Blue 2B. The staining depended to some extent upon the age of the rat and the size of the tumor. Four of the dyes tested in the present studies, however, gave satisfactory staining in mice and rats. The tumors were stained blue and the fat orange in rats and mice fed diets containing 5-benzylamino-9-dipropylamino, 5-benzylamino-9-dibutylamino, 5-(4-chlorobenzylamino)-9-dipropylamino or 5-(4-chlorobenzylamino)-9-dibutylaminobenzo[a]phenoxazine dyes.

5-naphthylamino- 9 -dialkylaminobenzo[a]-phenoxazine compounds behaved like phenyl derivatives in staining fat *in vivo*. A 6-membered nitrogen heterocyclic ring could also be substituted for the phenyl group without abolishing the *in vivo* fat-staining properties of the dyes.

The results of experiments carried out in the present study showed that a number of the benzo[a]phenoxazine dyes were soluble in various types of fat, but only those containing the 5-phenylamino group or those having the hydrogens of the 9-amino group replaced by alkyl radicals containing more than 2 carbon atoms stained fat *in vivo*.

Discussion. Certain benzo[a]phenoxazine dyes stained fat *in vitro*, but not all of them were capable of staining fat *in vivo*. While the available evidence does not permit of a definite conclusion as to the mechanism of *in vivo* fat staining, it appears probable that it is related to the basicity of the dyes in question. The 5-phenylamino compounds stained fat *in vivo;* the corresponding derivatives having highly acidic substituents on the benzene ring did not.

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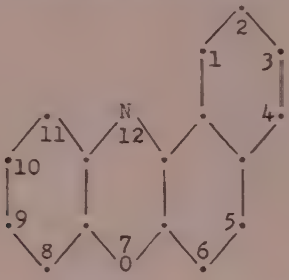
7. Crossley, M. L., Dreisbach, P. F., Hofmann, C. M., Turner, R. J., and Parker, R. P., Abstracts of papers presented at Am. Chem. Soc. Meeting, Cleveland, April, 1951, p17L, to be published.

TABLE I. Staining Effects of Benzo [a] phenoxazine Dyes.† Substituted 5.9-diamino derivatives.

A. Derivatives containing a phenyl group on the 5-amino nitrogen (salt or base indicated)



	Staining		
	Tumor	Fat	Urine
5-phenylamino-9-dimethylamino, chloride	0	Salmon	Pale green
5-(2-methylphenylamino)-9-dimethylamino, nitrate	Pale green	”	” ”
5-(3-methylphenylamino)- ” chloride	” ”	Pink	” ”
5-(4-methylphenylamino)- ” ”	Pink	Peach	” ”
5-(2-chlorophenylamino)- ” ”	0	Pink	0
5-(3-chlorophenylamino)- ” ”	0	Salmon	0
5-(4-chlorophenylamino)- ” ”	0	Peach	0
5-(2-hydroxyphenylamino)- ” ”	0	0	Pale green
5-(3-hydroxyphenylamino)- ” ”	0	0	0
5-(3-hydroxy-4-carboxyphenylamino)-9-dimethylamino, chloride	0	0	0
5-(2-methoxyphenylamino)-9-dimethylamino, chloride	Orchid	Pink	Pale green
5-(4-N-2-pyrimidyl-sulfamylphenylamino)-9-dimethylamino, chloride	0	”	” ”
5-phenylamino-9-ethylamino, chloride	Green blue	”	Blue
5-phenylamino-9-diethylamino, chloride*	Blue	Red	Blue green
5-(2-methylphenylamino)-9-diethylamino, nitrate	Violet	”	Green
5-(3-methylphenylamino)- ” ”	Pale violet	”	Pale green
5-(4-methylphenylamino)- ” chloride	Violet	”	” ”
5-(4-ethylphenylamino)- ” nitrate	”	”	” ”
5-(4-isopropylphenylamino)- ” ”	”	”	” ”
5-(4-t-amylphenylamino)- ” ”	0	Peach	” ”
5-(4-chlorophenylamino)- ” chloride	? pink	Red	” ”
5-(2-hydroxyphenylamino)- ” ”	Pale green	0	Bright green
5-(3-hydroxyphenylamino)- ” ”	Violet	0	Pale green
5-(4-hydroxyphenylamino)- ” ”	0	0	” ”
5-(2-hydroxy-5-chlorophenylamino)-9-diethylamino, chloride	0	0	” ”
5-(4-carboxyphenylamino)- ” ”	0	0	0
5-(2-methyl-4-chloro-5-nitrophenylamino)-9-diethylamino, base	0	Pink	Yellow
5-(2-biphenylamino)-9-diethylamino, chloride	0	Peach	0
5-(4-N-2-pyrimidyl-sulfamylphenylamino)-9-diethylamino, chloride	0	Pale violet	0
5-(4-methylphenylamino)-9-isopropylamino, nitrate	Violet	Purple	0
5-phenylamino-9-dipropylamino, nitrate	”	Red	Pale green
5-(2-methylphenylamino)-9-dipropylamino, nitrate	”	”	” ”
5-(3-methylphenylamino)- ” ”	”	”	” ”
5-(4-methylphenylamino)- ” bromide	”	Rose	0
5-(4-methylphenylamino)- ” phosphate	”	Scarlet	Pale green
5-(4-methylphenylamino)- ” sulphate	”	Rose	Blue
5-(4-ethylphenylamino)-9-dipropylamino, nitrate	Pale violet	Red	Pale green
5-(4-ethylphenylamino)- ” sulphate	” ”	Rose	0
5-(4-isopropylphenylamino)- ” nitrate	” ”	Red	0
5-(4-isopropylphenylamino)- ” phosphate	Violet	”	Pale green
5-(4-chlorophenylamino)- ” nitrate	0	”	” ”
5-(N-ethyl-N-phenylamino)- ” ”	? violet	”	0
5-phenylamino-9-dibutylamino, nitrate	0	Pink	0
5-(4-methylphenylamino)-9-dibutylamino, nitrate	0	”	0
5-(4-methylphenylamino)- ” base	0	”	0
5-phenylamino-9-diamylamino, base	Pink	”	0

		Staining	
	Tumor	Fat	Urine
5-phenylamino-9-dihexylamino, base	0	0	0
5-(4-methylphenylamino)-9-methylpropylamino, base	0	Red	Yellow
5-phenylamino-9-ethylpropylamino, nitrate	Violet	”	Green
5-(4-methylphenylamino)-9-ethylpropylamino, nitrate	Pale violet	Scarlet	Pale green
5-phenylamino-9-butylpropylamino, nitrate	” ”	Red	” ”
5-(2-methylphenylamino)-9-butylpropylamino, nitrate	” ”	”	Yellow
5-(4-chlorophenylamino)- ” ”	Pink	”	Pale green
5-phenylamino-9-(4-morpholinyl), chloride	0	Orange	0
5-(4-methylphenylamino)-9-(4-morpholinyl), chloride	Green blue	”	0
5-(4-methylphenylamino)-9-diethylamino-10-methyl, chloride	Violet	Russet	Pale green
5-phenylamino ” -11-methyl, nitrate	0	Pale pink	Orchid
5-(2-methylphenylamino)- ” ” ”	0	Pink	0
5-(4-methylphenylamino)- ” ” ”	Pale blue	Red	Pale green
5-(4-methylphenylamino)- ” ” base	Blue	Rose	” ”
5-(4-chlorophenylamino)- ” ” nitrate	0	Pink	Pale violet
5-phenylamino-9-ethylamino-10 (or 8)-methyl, nitrate	0	Salmon	Pale green
5-(4-methylphenylamino)-9-ethylamino-10 (or 8)-methyl, nitrate	?	Red	” ”
B. Derivatives containing a group on the 5-amino nitrogen, other than a phenyl group (salt or base indicated)			
5-(1-naphthylamino)-9-diethylamino, chloride	? blue	Red	0
5-(2-naphthylamino)- ” ”	0	Pink	0
5-benzylamino-9-dipropylamino, chloride	Blue	Orange	Pale blue
5-(4-methylbenzylamino)-9-dipropylamino, chloride	Pale blue	Pink	Blue
5-(4-chlorobenzylamino)- ” ”	Blue	Salmon	”
5-benzylamino-9-dibutylamino, chloride	Deep blue	”	Pale blue
5-(4-methylbenzylamino)-9-dibutylamino, chloride	Blue	Orange	” ”
5-(4-chlorobenzylamino)- ” ”	”	Salmon	” ”
5-benzylamino-9-diamylamino, chloride	”	”	? green
5-(4-methylbenzylamino)-9-diamylamino, nitrate	”	”	Pale green
5-(4-chlorobenzylamino)- ” chloride	Pale blue	Peach	” ”
5-benzylamino-9-dihexylamino, chloride	Blue	Russet	” ”
5-(4-methylbenzylamino)-9-dihexylamino, nitrate	”	Salmon	” ”
5-(4-chlorobenzylamino)- ” chloride	Pale blue	”	” ”
5-(2-pyridylamino)-9-diethylamino, chloride	Pale green	Rose	” ”
5-(2-pyridylamino)-9-dipropylamino, nitrate	0	”	Green
5-(2-pyrazinylamino)- ” ”	Violet	Purple	0
5-(2-pyridylamino)-9-dibutylamino, nitrate	”	Rose	Pale green

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* N-Phenyl Nile Blue A Chloride.

† The expressions used to designate color in the tables are based on the visual observation of the investigator.

Microscopical examination of the stained fatty tissue showed that the color was confined to the fat globule, the surrounding cytoplasm remaining uncolored. Fat globules in macrophages and supporting tissue were stained the same color as those in the fatty tissue. As may be seen in the tables, the color exhibited by the fatty tissue in the treated animals depended upon the particular dye ingested. The majority of the dyes stained fatty tissue tones or orange and red, although a few stained the fat tones of pink and purple. The color which appeared in the tumors of the treated mice also varied depending upon the dye ingested; the color of the tumors was not the same as that of the fat in the same animal. In general tumor tissue became stained blue or tones of blue mixed with red or with yellow.

Summary. (1) 5-phenylamino-9-dialkylaminobenzo[a]phenoxazine dyes stained fat *in vivo* when administered to mice in their food. (2) The fat staining properties of the dyes were not materially altered by substituents on the phenyl nucleus other than those of highly acidic nature, or by substitutions of the naphthyl group and certain nitrogen heterocyclic rings for the phenyl group. The 5-phenylamino-9-morpholinyl derivatives also stained fat *in vivo*. (3) 5-benzylamino-9-dimethylamino- and diethylaminobenzo[a]phenoxazine dyes did not stain fat *in vivo*, although they did stain tumor tissue. The corresponding dyes in which the alkyl group on the 9-nitrogen contained more than 2 carbon atoms stained fat *in vivo*. These dyes also stained tumor tissue *in vivo*.

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Studies of Proteolytic Enzyme Systems in Patients with Emotional Disorders.* (19087)

JAMES S. L. JACOBS, PHILIP M. WEST, AND CLINTON E. TEMPEREAU.
(Introduced by C. M. Carpenter.)

From the Departments of Psychiatry and Investigative Medicine, Long Beach Veterans Administration Hospital, Long Beach, and Departments of Infectious Diseases and Biophysics, School of Medicine, University of California, Los Angeles.

Studies of protein metabolism have revealed well-defined deviations in the concentrations of certain protein-splitting enzymes in the sera of patients with cancer(1-3). This report concerns the existence of analogous enzymatic disturbances in the presence of emotional disorders. The enzymes in question are accompanied in the blood by excesses of their inhibitors. Measurements of the latter permit valid deductions referable to the particular enzyme systems. Chymotrypsin-inhibitor is one element of an enzyme system which correlates with the rate of protein destruction. For example, abnormal concentrations have been noted in the presence of cancer, infections, physical trauma and wasting diseases. The relief from anxiety afforded by prefrontal lobotomy(4) for the alleviation of intractable pain of cancer, is often followed by significant reductions in abnormally increased concentration of chymotrypsin-inhibitor. Electric convulsive therapy provides comparable results. Therefore it becomes important to know the effect of emotional status alone upon the titer of chymotrypsin-inhibitor. Under certain circumstances its concentration in the serum correlates with the intensity of emotional reaction. In a series of 102 patients, the degree of anxiety experienced by each was estimated clinically (Fig. 1.) A graded scale of 5 divisions was employed: absence of anxiety and panic represented the extreme categories; slight, moderate and severe anxiety were defined in the intermediate groups. Only schizophrenic psychotics, conversion hysterics and patients with acute anxiety states were included. A direct correlation between the degree of anxiety and elevations of chymotrypsin-inhibitor concentrations was found to exist in the last group alone. Patients with conversion hysteria exhibited no such relationships, and furthermore seldom demonstrated more than slight elevations in titer. On the other hand, schizophrenic psychotics showed highly variable enzymatic changes, some of which were directly related to the level of anxiety, while the majority were not. But, in this group high titers *were* obtained.

It then became imperative to investigate the possibility that the changes in protein metabolism might be associated with variations in adrenal function. The demonstration that the urinary excretion of the proteolytic enzyme, uropepsin, is an appropriate measure of adrenal activity, the titer directly paralleling adrenal function(5), provided a satis-

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* Reviewed in the Veterans Administration and published with the approval of the chief medical director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinions or policy of the Veterans Administration.

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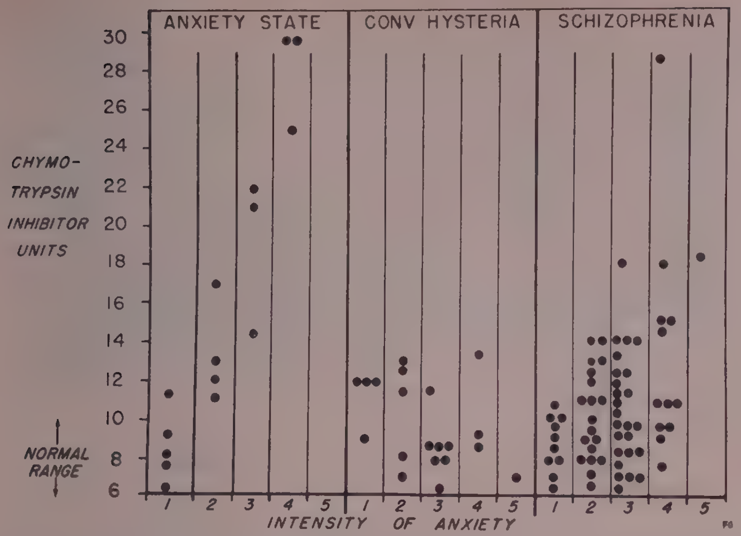

FIG. 1. Concentration of chymotrypsin inhibitor in the sera of 102 patients classified as anxiety state, conversion hysteria and schizophrenia. Each group is sub-classified according to the intensity of anxiety on a 5 point scale. (Chymotrypsin inhibitor determinations are recorded on a more sensitive scale than that described in ref. 1 and 2.)

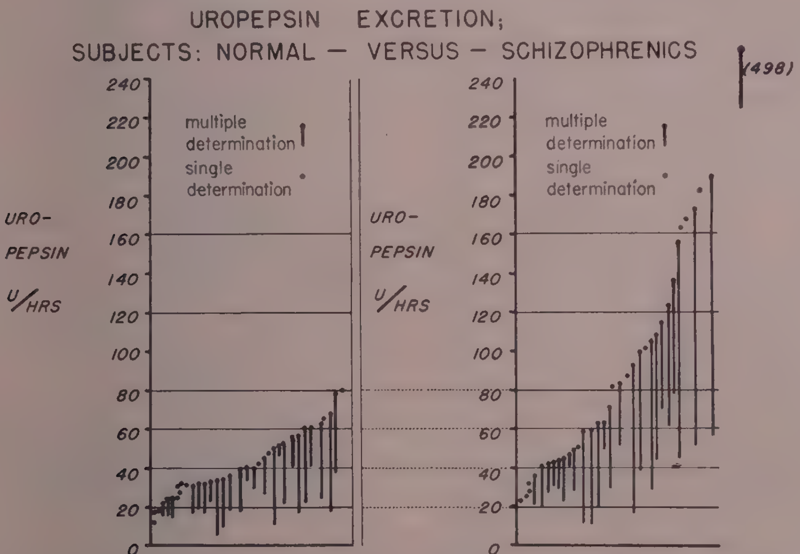

FIG. 2. Uropepsin excretion of 36 normal and 36 schizophrenic subjects. Each line indicates the degree of variation in a single subject; a dot signifies a single uropepsin determination.

factory method for this purpose. Uropepsin determinations were performed on the urines of 36 normal individuals and 36 schizophrenic psychotics; 1-15 tests were completed in each case (Fig. 2). It was found that in general the urines of schizophrenic psychotics contained larger quantities of uropepsin than did those of normals; sometimes remarkable quantities were noted. Furthermore, the output among schizophrenics was far more variable than among the controls. The data indicate that in the presence of a schizophrenic psychosis increased and highly variable adrenal activity is a common occurrence, and is apparently unrelated to psychomotor activity.

Summary. These investigations imply that emotional disorders are accompanied by changes in protein metabolism and adrenal activity. The protein metabolic disturbances are generally in the direction of increased catabolism, being well correlated with emotional response when the disorder is essentially an anxiety reaction to acute stress, less well correlated in schizophrenic psychosis, but sometimes still pronounced and, finally, lacking in correlation in the presence of conversion hysteria. Furthermore, a comparison of schizophrenic psychotics with normal controls reveals that the former exhibit a generally higher and more variable state of adrenal activity. It is hoped that these ostensibly unrelated sets of data may eventually become integrated with a coherent concept of individual reactions to stress.

The authors wish to express their appreciation for the technical assistance of Jessamine Hilliard, Virginia Hansen, Willard Keye, and Bronnetta Scott.

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Rapid CO_2 Determination with the Beckman O_2 Analyzer.* (19088)

VIVIAN G. BEHRMANN AND FRANK W. HARTMAN.

From the Department of Laboratories, Henry Ford Hospital, Detroit.

The current daily hospital routine, which includes Tissot basal metabolism, maximum breathing capacity and exercise tests, requires from 40 to 60 expired air analyses in duplicate. The classical Haldane technic has been the method of choice for many years. Since 1947, efficiency has been increased through the innovation of O_2 analysis, based upon the paramagnetic qualities of O_2 so ingeniously devised by Pauling(1). Consequently, CO_2 has been determined as usual by Haldane, with only a complete duplicate analysis as a check on 1 out of every 10 determinations on the Beckman O_2 analyzer. To further minimize Haldane analysis, the search for a cheap, rapid, CO_2 method accurate enough for this purpose has been our objective in recent years. Infra-red spectroscopy(2,3) and the mass spectrograph(4) are especially suited to accurate, quick, CO_2 determination in continuous analysis but are elaborate and expensive. Although a variation of the simpler and cheaper venturi meter method(5) has been tried and a conductivity apparatus(6,7), which we have built into a constant temperature chamber, is now in use, the principle of

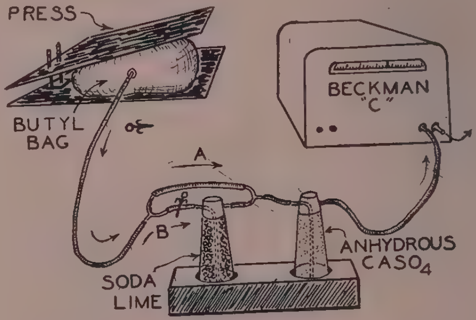

FIG. 1.

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* Presented before the Am. Physiol. Soc., May 1, 1951.

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FIG. 2.

the CO_2 method to be presented in this report was disclosed rather fortuitously as a consequence of our early experience in magnetic O_2 analysis. In the conversion from the classical Haldane Hg sampling tubes to bags, suitable for paramagnetic gas analysis, a gradual apparent increase in the O_2 values, resulting from CO_2 loss through the rubber, was observed. Transfer to synthetic butyl bags, which are practically impervious to CO_2 (approximate loss of 0.01-0.03% CO_2/24 hr) allowed O_2 values consistent with Haldane results. This experience suggested the idea that CO_2 calculation might be possible if the apparent O_2 increase on complete CO_2 removal were significant. Since this proved to be true, the feasibility and accuracy of this method has been tested.

The procedure is shown diagrammatically in Fig. 1. In step A the gas is pressed from its butyl bag, at an optimum flow rate of 200 cc per minute for 2½ minutes through anhydrous $CaSO_4$ to the Beckman analyzer, (C model) resulting in the reading of the O_2 tension in mm Hg partial pressure, which is readily converted to O_2 percentage. Without delay the O_2 percentage after CO_2 removal (step B) is similarly determined by diverting the gas through soda lime and anhydrous $CaSO_4$ into the analysis chamber. The procedure was facilitated by a continuous barometric pressure record (Baroscribe), a 2½-minute timer, a small lamp, and a magnifocuser (a binocular loupe, which magnifies 3x) to increase accuracy of scale reading, as depicted in Fig. 2.

The efficiency of ascarite, KOH solution (17%) and soda lime in absorption bulbs of variable size and shape was tested. Ascarite, though highly effective as a quantitative CO_2 absorbent, tended to solidify hindering the flow rate. KOH solution was also satisfactory, but to avoid the damage which might result if the alkaline reagent entered the gas chamber, the equally efficient and cheap soda lime (mesh No. 4-8, moisture content 4-19%) was preferable. Anhydrous $CaSO_4$ (indicating drierite, mesh No. 8) proved to be an efficient agent for moisture removal. The drierite (100 g) was changed when two-thirds of it had turned pink, usually after 100-150 determinations. The soda lime (100 g) remained effective for approximately 450-500 CO_2 analyses.

CO_2 calculation was made by the following simple relationship: Let $A = O_2\%$; $B = O_2\%$ after CO_2 removal; $B = 100\ A/100 - CO_2\%$; $CO_2\% = 100\ (B-A)/B$.

Data was accumulated in duplicate on 300 expired air samples, using Haldanes, the Beckman E-2 model and the Beckman C model O_2 analyzers. Considering that a small CO_2 percentage is determined by the difference between two relatively large O_2 percentages, the results have proved to be quite consistent. Results on 10 consecutive samples are shown in Table I. On the basis of averages, the average deviation from the Haldane is practically identical for both models (0.065 for C and 0.069 for E-2) but the variation between duplicate CO_2 values is definitely greater in the E-2 data. It is true that the accuracy of the CO_2 value depends

TABLE I. Consecutive CO_2 % Determinations in Duplicate.

Sample No.	Haldane	Avg	Beckman C model	Avg	Beckman E-2 model*	Avg
1	1.93	1.91	1.99	1.99	1.89	1.81
	1.89		1.99		1.73	
2	2.43	2.41	2.43	2.46	2.54	2.46
	2.39		2.49		2.38	
3	3.19	3.18	3.19	3.16	3.24	3.12
	3.17		3.13		3.07	
4	3.46	3.49	3.43	3.43	3.49	3.52
	3.52		3.43		3.54	
5	2.80	2.78	2.83	2.86	2.83	2.80
	2.76		2.89		2.77	
6	3.71	3.70	3.74	3.69	3.85	3.88
	3.69		3.64		3.90	
7	3.58	3.57	3.50	3.47	3.67	3.65
	3.55		3.44		3.62	
8	3.85	3.84	3.67	3.69	3.81	3.75
	3.83		3.70		3.69	
9	3.17	3.18	3.09	3.11	3.08	3.17
	3.19		3.12		3.26	
10	3.26	3.24	3.18	3.21	3.18	3.24
	3.22		3.24		3.29	

* Experience with the E-2 model has been less satisfactory, as indicated by the wide variation between duplicates.

upon the care with which the O_2 readings are made. Therefore, it follows that the sensitivity as well as the percentage range of the Beckman instrument used are also determining factors in the degree of accuracy obtainable. The Beckman E-2 model, a null instrument, requires a potentiometric return of the light beam to zero. The technician must adjust

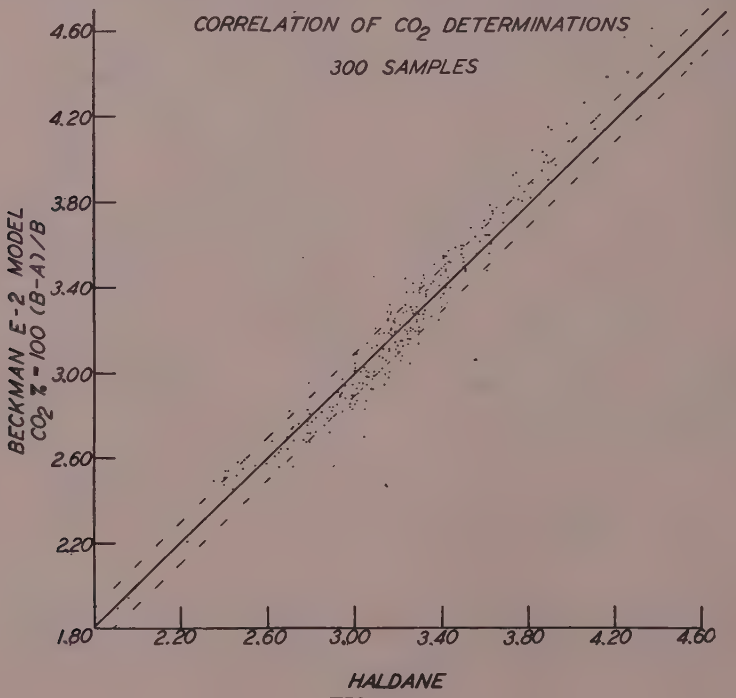

FIG. 3.

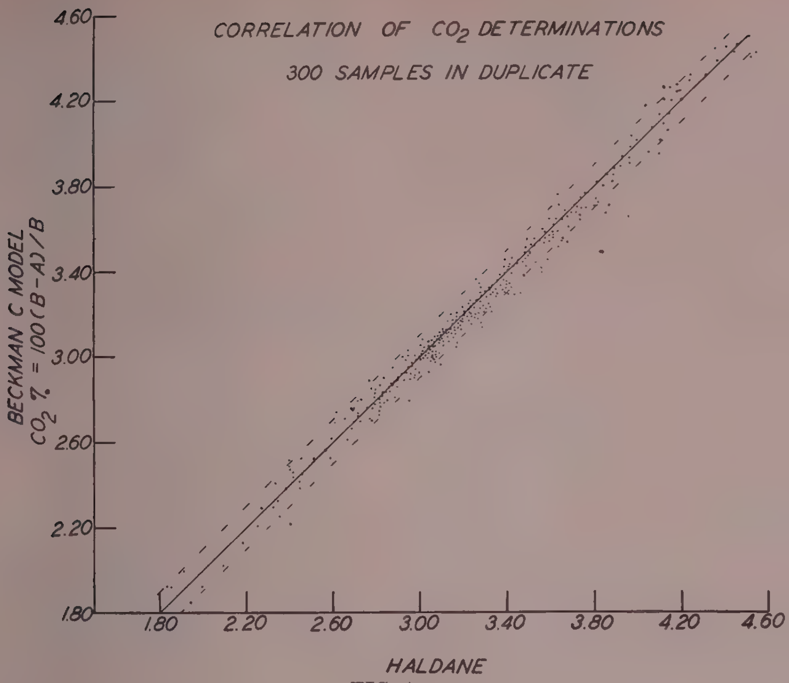

FIG. 4.

the beam to zero and take a reading. This two-step procedure is subject to more error than the simple scale reading of the deflected beam as in the Pauling type C model. Secondly, the E-2 model range of 0-25% is approximately 3½-4 times the range of the C model (110-160 mm Hg). A narrower range on the E-2 model (15-25% or 12-22%) should improve the accuracy. A comparison of results on the E-2 model (Fig. 3) and on the C model (Fig. 4) illustrates this point.

A statistical analysis[†] of 20 random CO_2 samples, determined by the three instruments was performed using Student's rule of "t". A simple comparison is made in Table II. The Haldane has the best "t" value, indicating that it offers the best accuracy. The C-model Beckman has only a slightly lower "t" value showing that the agreement is excellent. Data on the E-2 model has the lowest "t" value, as one would expect. It is important to note that a determination of the "t" value of the difference between the means points to the fact that all 3 methods are equally valid from a statistical point of view. Here, too, the best agreement is between the Haldane and the C model; the next best agreement is between the Haldane and the E-2 model; while the C and the E-2 differ more widely. This occurs because the E-2 values tend to run on the high side of the Haldane and the C values on low side.

Conclusion. This method offers a simple

TABLE II. Statistical Comparison, 20 Random CO_2 Samples in Duplicate.

	Mean value ± S. E.	t value	t value of difference between means	
Haldane	3.303 ± .90	36.518		
			.183	
Beckman C	3.286 ± .90	36.367		.350
			.474	
Beckman E-2	3.351 ± .103	32.525		

[†] Equations employed in the statistical analysis were: $\alpha = \sqrt{\frac{\Sigma d^2}{n-1}}$; $SEm = \frac{\alpha}{\pm\sqrt{n}}$; $t = \frac{mean}{SEm}$ and

t of difference between means $= \frac{mean-mean}{\sqrt{SEm^2 + SEm^2}}$.

quick technic for separate expired air samples. Both CO_2 and O_2 analysis may be completed within 5 minutes as compared to 15-20 minutes by Haldane. Ninety-five per cent of the duplicate CO_2 analyses as determined with the Beckman C model (110-160 mm Hg range) will agree within 0.10% with duplicate Haldane data. A Beckman instrument of a narrower range and/or greater sensitivity would improve this accuracy. In our experience the Beckman C model (110-160 mm Hg range) allows better results than does the more elaborate and expensive E-2 model.

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Activation of Pyruvate Oxidation in Tumor Mitochondria by Diphosphopyridine Nucleotide.* (19089)

Charles E. Wenner,† Morris A. Spirtes, and Sidney Weinhouse.
(Introduced by S. P. Reimann.)

From the Lankenau Hospital Research Institute and Institute for Cancer Research, Philadelphia.

Previous studies from our laboratory have disclosed that intact tumor cells (as exemplified by slices of various transplanted mouse neoplasia) are able to oxidize glucose and fatty acids to completion via the citric acid cycle(1,2). It therefore appeared that previous failures to observe oxidation of pyruvate or oxalacetate in homogenates of tumors(2-6) were due to loss of some labile factor in homogenization rather than to lack of oxidative capacity of the tumor cell.§ In an investigation of this matter it was found that good oxygen uptake by whole homogenates of tumors could be achieved in the presence or absence of substrates if rather high concentrations of diphosphopyridine nucleotide (DPN) were added; some preliminary results of these experiments have already been reported(1). The necessity for DPN in oxidation processes of tumors has been more strikingly demonstrated in experiments with isolated mitochondria, and these are reported in the present communication.

Methods and materials. The tumors used in this study are maintained by subcutaneous implantation in adult mice of the A or C3H strains. When tumors attained a size of 1 cm the animals were sacrificed by decapitation. Mitochondria of normal and neoplastic tissues were obtained by following exactly the procedure of Schneider(7).‡ The washed mitochondria were suspended in isotonic sucrose and kept refrigerated until used. All operations were carried out at <5°C. The tissue suspension was added to chilled Warburg vessels containing the ions, factors and substrates designated in Table I. The pyruvate and malate were commercial products. Diphosphopyridine nucleotide was obtained from various commercial sources, and assayed about 40% by the enzymatic procedure of Racker

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[†] This work will constitute part of a thesis to be presented by C. E. Wenner to the Graduate School of Temple University in partial fulfillment of the requirements for the Ph.D. degree.

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[§] Potter and Lyle(6) have recently reported that pyruvate oxidation will occur in tumor homogenates in the presence of fluoride.

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TABLE I. Oxidation of Pyruvate by Mitochondria of Mouse Tumors. Experiments run 1 hr in air at 38°. The basic system contained the following substances in the designated final concentrations: $MgSO_4$, 3×10^{-3} M.; fumarate, 7×10^{-5} M. (as a primer); adenosine triphosphate (ATP) 2×10^{-3} M.; cytochrome C. 4×10^{-6} M.; phosphate buffer, 7.4 pH, 6×10^{-3} M.; and .6 ml of the mitochondria suspension representing 300 mg of original tissue. To this there was added diphosphopyridine nucleotide (DPN) 1.9×10^{-3} M. or sodium pyruvate, 8×10^{-3} M., or both; and the final volume made to 1.6 ml. Values are in μl per hr.

Tissue	Additions to basic system			
	None	DPN	Pyruvate	DPN + pyruvate
C3H hepatoma	23	62	22	187
A hepatoma	13	28	97	176
Mammary carcinoma	2	21	10	154
Sarcoma 37	22	43	21	77
Rhabdomyosarcoma	—	22	10	66

(8). Cytochrome C and ATP were commercial products. After an initial 7-minute equilibration, the flasks were shaken one hour at 38° with air as the gas phase, and oxygen uptake measurements made by the usual methods. Nitrogen was determined by a micro-Kjeldahl procedure(9).

Results. The effect of DPN in activating pyruvate oxidation by tumor mitochondria is demonstrated in Table I, in which data are given for 5 neoplastic tissues. In a system containing all of the known cofactors required for oxidation of pyruvate, oxalacetate, other citric acid cycle components, and fatty acids by mitochondria of normal tissues(3,7,10,11) oxygen consumption was very low, and was not stimulated appreciably by the addition of pyruvate. This observation is essentially the same as reported by Potter *et al.*, for pyruvate and oxalacetate oxidation in homogenates and in mitochondria of several transplanted rat tumors(3,4). Only one apparent exception was noted in the case of the A strain hepatoma, which displayed appreciable oxygen uptake on the addition of pyruvate alone. When DPN in a final concentration of 2×10^{-3} M was added to the fortified mitochondria, small increments in oxygen consumption occurred, which could be attributed to DPN activation of endogenous metabolites; however, when both pyruvate and DPN were present together high levels of oxygen consumption were observed, which were of similar magnitude to those obtained with mitochondria of normal liver(5,11).

The rigid requirement of added DPN for oxidation of pyruvate by mitochondria of C3H mouse hepatoma is displayed by Table II, in which there is recorded a study of the effects of omission of various factors from the system. Diphosphopyridine nucleotide was the only substance whose omission lowered oxygen consumption to an almost negligible value. The approximately 30% decrease in oxygen consumption on omission of fumarate is a reflection of the well-established priming effect of small amounts of Krebs cycle components on oxidation in mitochondria(11) and can be taken as further evidence for occurrence of the citric acid cycle in mitochondria of neoplastic tissues. The drop in activity on omission of ATP is in harmony with

TABLE II. Effects of Omissions on Pyruvate Oxidation by Mitochondria of Mouse Hepatoma. The complete system was that of Table I with DPN and pyruvate present. Values are in μl per hr.

Conditions		O_2 consumption
Complete system		187
DPN and pyruvate omitted		23
DPN	”	22
Pyruvate	”	62
Fumarate	”	112
ATP	”	117
Cytochrome C	”	133

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‡ Owing to a defective transformer, which was not discovered until most of our experiments were completed, the centrifugations of mitochondria were conducted at 5000-6000 g instead of the recommended 8000 g. This resulted in recoveries of mitochondria which were about 60% of the amounts obtained at the higher speed. This fraction contained no nuclei or intact cells which could be seen microscopically in stained preparations. Had all of the mitochondria been sedimented our observed oxygen uptakes, recorded in Tables I and II, would have been considerably higher, but the figures in Table III, which are on a per mg nitrogen basis would not have been changed.

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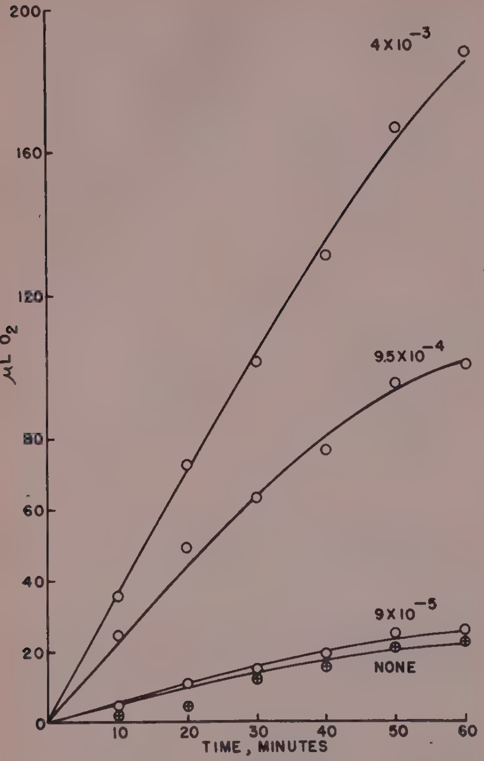

FIG. 1. DPN levels and oxygen consumption by suspensions of hepatoma mitochondria. Conditions are as given in Table I.

previous findings concerning its stimulating effect on oxidation in liver mitochondria (5,11). Apparently the ATP requirement in tumor mitochondria is not so high in the presence of DPN, since omission of the former resulted in only a 30% drop in activity. This is also in accord with previous observations of Lehninger and Kennedy(10), and Potter *et al.*(4) who found that DPN could at least partially replace the ATP requirement for oxidations in normal cell particles. The loss of some activity on omission of cytochrome C may be considered as a result of a deficiency in tumor mitochondria of this electron transport factor(12), however, here again a similar reduction in octanoate oxidation has been reported with respect to omission of cytochrome C in liver mitochrondria(11).

The relationships between oxygen uptake and DPN level in the oxidation of pyruvate by mitochondria from the C3H hepatoma is revealed in Fig. 1. Relatively high concentrations of DPN of the order of 0.002 M are required for maximal activation; further increase in DPN concentration did not raise oxygen consumption above the level shown in this figure. In the experiments of Potter *et al.*(3,4) on oxalacetate and pyruvate oxidation in homogenates of rat tumors, no effect of DPN was observed; however, the highest concentrations used were only .0003 M. It is evident from Fig. 1 that this concentration was too low for appreciable activation in our experiments. It may be presumed, since the two sets of experiments were carried out under almost identical conditions except for DPN concentration, that Potter *et al.* would also have been able to activate oxalacetate oxidation if their DPN concentration had been higher. The use of 40% DPN in our experiments raises the question whether its effects are actually due to some contaminant. This seems unlikely for two reasons. First, the effects obtained with different samples were proportional to the DPN content as determined enzymatically(8); and, second, occasional checks with 90% DPN (obtained from General Biochemicals Inc.) gave the same results as the less pure specimens.

When these studies disclosed the capacity of tumor mitochondria for pyruvate oxidation, it was of interest to make comparisons with mitochondria of normal tissues. It was already evident from Warburg's experiments (13) that the respiratory activity of tumor slices was somewhat low when compared with metabolically active normal tissues such as liver and kidney; and essentially the same finding was reported in a recent isotopic tracer study(2). Inasmuch as it is well established that at least a major portion of the oxidative activity of cells is concentrated in the mitochondria(14) it appeared that such differences as exist between normal and neo-

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TABLE III. Pyruvic and Malic Oxidase Activities of Mitochondria of Normal and Neoplastic Tissues.

Tissue	O_2 uptake, μl/hr/mg N Pyruvate	Malate
Neoplastic		
A-strain hepatoma	315 318 246	314
C3H strain hepatoma	312	—
Rhabdomyosarcoma	150	159
Sarcoma 37	292	230
Normal		
Kidney (C3H mouse)	292	278
Liver (A mouse)	252 224 169	145
Kidney, rat	380* 642†	
Liver, ”	220* 394†	
” ”	141‡	

* Data of Potter, *et al.*(16). Substrate, oxalacetate and pyruvate.

† Data of Schneider and Potter(5). Substrate, oxalacetate and pyruvate.

‡ Data of Kennedy and Lehninger(11). Substrate, pyruvate plus oxalacetate.

plastic tissue in oxidative capacity could be due to the lower content of mitochondria in tumors(14). Some support for this view is found in recent data of Schneider and Hogeboom(15) who reported that although the total nitrogen content of a mouse hepatoma was three-fourths that of mouse liver, the tumor mitochondrial nitrogen constituted only 13% of the total cell nitrogen, whereas liver mitochondrial nitrogen was 23% of the total. Thus, if a particular enzymatic activity is found only in mitochondria and is proportional to the nitrogen content thereof, the apparent enzymatic activity of the normal tissue will be $\frac{23}{13} \times \frac{100}{75} = 2.4$ times that of the tumor.

To compare the pyruvic (and malic) oxidative activities of tumor mitochondria with those of normal tissues, separate aliquots of mitochondria were simultaneously tested under the conditions outlined in Table I, and assayed for nitrogen; and oxidative activity was calculated on the basis of the nitrogen content. Although liver mitochondria generally do not need added DPN for maximal activity, it was added to all flasks in order to make the experiments comparable. The data are given in Table III. Three of the four tumors gave essentially equal pyruvic oxidative activities per mg of mitochondrial nitrogen, and these were fully as high as those displayed by mouse liver and kidney, the only exception being rhabdomyosarcoma. Whether this tumor is actually different in this respect from the others is now under study. Malic oxidative activities were, on the whole, in the same range as those of pyruvate.

In several instances it was possible to compare these values with data from the literature. Potter, *et al.*(16) reported graphic data (Fig. 1) for oxalacetate oxidation by mitochondria of rat liver and kidney, and similar data were reported by Schneider and Potter(5) (Tables I and II). These were recalculated on a per mg nitrogen basis by assuming, from Schneider and Hogeboom's data(15), that normal tissues contain 3% of nitrogen and that the mitochondria contain 25% of the total cell nitrogen. A similar calculation was made for a single experiment reported by Kennedy and Lehninger(11) (Table IV). These values are included in Table III. When one considers all the differences in techniques and conditions between these various experiments the agreement is surprisingly good.

We conclude from these results that mitochondria of neoplastic cells have the same capacity to oxidize pyruvate and malate as those of normal tissues. It does not necessarily follow that all of the enzymes concerned with the reactions of the citric acid cycle or those involved in electron transport through the pyridine nucleotides, flavoproteins and cytochromes occur in the same amounts in both types of mitochondria. Indeed, according to experiments of Schneider and Hogeboom(15) mitochondria from mouse hepatoma have less than half as much succinic oxidase and cytochrome oxidase activity as mitochondria from normal mouse liver. It does appear, however, that mitochondria of normal and neoplastic tissues do have essentially the same makeup of oxidative enzymes and also it would appear that in the

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16. Potter, V. R., Lyle, G. G., and Schneider, W. C., *J. Biol. Chem.*, 1951, v190, 293.

complex system of reactions resulting in pyruvate oxidation the same step is rate-controlling in both types of mitochondria.

Discussion. The fact that DPN must be added to tumor mitochondria to bring about oxidation of pyruvate is not surprising. It is more surprising, in fact, that it is apparently not necessary for oxidations by mitochondria of normal tissues, when one considers the important role of this soluble coenzyme in at least one step of the citric acid cycle, *viz.* malic oxidation, and probably also in pyruvate and α-ketoglutarate oxidation(17). However, a DPN requirement can be demonstrated under certain conditions. Lehninger (18) has shown that washed sedimentable liver particles which lost their activity on "ageing" could be reactivated by addition of DPN; and Potter, *et al.* and Kennedy and Lehninger(4,11) have pointed out that DPN can partially replace the adenine nucleotide requirement for mitochondrial oxidations. Further evidence of a DPN requirement in oxidations by kidney homogenates has been provided by Pardee and Potter(19), who found that loss in oxidative activity is paralleled by a decrease in particle-bound DPN; and that further addition of DPN would partially restore oxidative activity.

In an extensive study of the "cyclophorase" system of rabbit liver and kidney, the active component of which is the mitochondrial fraction, it was found by Green and members of his laboratory that mild physical or chemical treatment resulted among other changes in a loss of oxidative activity which could be regained by addition of DPN(20-22). It was concluded that the pyridinoproteins occur normally in a conjugated form in which the nucleotide is tightly bound to its apoenzyme; and that the effect of the various treatments was to dissociate the pyridine nucleotide. Whether or not one accepts this explanation, these studies have indicated that the nucleotide is present in normal mitochondria in a form which is not freely dissociable, and that alterations can easily be produced which create a DPN requirement but which do not otherwise impair the integrity of the particles. On this basis it might be assumed that the tumor mitochondrion differs from its normal counterpart by an excessive "fragility," so that the process of homogenization is sufficient to bring about changes resulting in the DPN requirement. The ease with which tumor mitochondria lose DPN may be related to the relative content of oxidized and reduced forms of the nucleotide(1). This point is now under investigation.

Another possibility is that tumors may be extremely active in splitting DPN. Although direct studies of this point have not been carried out, to our knowledge, Potter and Lyle(6) have recently shown that in comparison with normal tissues several rat tumors displayed an excessive rate of organic phosphate breakdown and have concluded that the primary reason for the loss in oxidative activity in tumor homogenates is an unusually high rate of destruction of high energy phosphate bonds, thus preventing maintenance of a sufficiently high level for oxidative activity. In support, these authors cited experiments in which oxidation was maintained in tumor homogenates if fluoride was added to minimize splitting of organic phosphate linkages. It is interesting in this connection that fluoride has been reported by Huennekens and Green (20) to stabilize the kidney cyclophorase system so as to make DPN addition unnecessary for oxidative activity.

Further work is obviously needed to clarify the nature of the DPN requirement for oxidation of pyruvate by tumor mitochondria; it seems apparent now, however, from the present study and from recent results of Potter and Lyle(6) that tumor mitochondria do not differ fundamentally from their normal counterparts in the enzymatic mechanisms of

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21. Huennekens, F. M., and Green, D. E., *Arch. Biochem.,* 1950, v27, 428.

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pyruvate oxidation.

Summary. Suspensions of mitochondria of various transplanted mouse tumors are able to oxidize pyruvic acid if the system, containing Mg ions, ATP, cytochrome C, and priming concentrations of fumarate, is fortified with diphosphopyridine nucleotide. The concentration of the nucleotide required for maximal activation is 0.002M. When mitochondria of tumors were compared with those of normal tissues it was found that on a per mg nitrogen basis pyruvate oxidation was similar in magnitude in both types of mitochondria.

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Metabolism of Plasma Substitutes II. Oxypolygelatin (OPG) (19090)

IRVING GRAY AND EDWIN J. PULASKI.
(With the technical assistance of Joanne A. Wilcox.)
From the Surgical Research Unit, Brooke Army Hospital, Brooke Army Medical Center, Fort Sam Houston, Texas.

The use of gelatin solutions as substitutes for plasma have long been investigated(1,2). Although gelatin appears to fill the requirements for a practical plasma expander, the main difficulty lies in its high gel point. Recently, Campbell and co-workers(3) have developed a modified gelatin, oxypolygelatin, (OPG) which appears to overcome this particular fault.

This paper is a report of the studies on the metabolism of this new plasma expander. Changes in the urinary dextrose to non-protein nitrogen ratio (D/N) of the phlorizinized, starved dog were used as an index of metabolism of OPG. The details of these experiments have been reported in the first paper of this series(4). The results are summarized in Table I. In each case, during the 24-hour period following the infusion of 200 ml OPG in physiologic saline, we have a marked increase in the D/N ratio, well beyond the limits of the pre-infusion spread.

TABLE I. Changes in D/N Ratio After Infusion of 200 ml of 5% Solution of OPG in Physiological Saline.

Dog No.	Pre-infusion avg	Range	0-24 hr post-infusion D/N
1	1.39	1.23-1.69	2.4
3	2.76	2.17-3.75	4.42
6	2.27	2.17-2.30	3.34
48	3.47	3.30-3.91	5.29

Since the phlorizinized starved dog stabilizes at a constant D/N ratio, a change in this value reflects changes in the glucose or nitrogen metabolism. Because phlorizin acts by preventing the reabsorption of glucose by the tubules, an increase in D/N ratio indicates an increase in the excreted glucose relative to the excreted nitrogen. As was pointed out in the previous discussion(4), it is felt that glycogen can be ruled out as a source of the increased glucose as can the plasma proteins. Therefore, the highly probable possibility left which might cause this rise in the D/N ratio is the metabolism of OPG to glucose.

Summary. Evidence is presented to show the metabolism of OPG to glucose by the phlorizinized starved dog.

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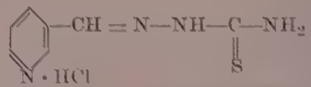
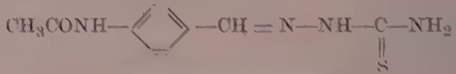
Spectrophotometric Determination of Nicotinaldehyde Thiosemicarbazone in Blood. (19091)

Alex E. Motchane and Wilbur M. Benson. (Introduced by S. H. Rubin.)
From the Departments of Physical Chemistry and Pharmacology, Hoffmann-La Roche Inc., Nutley, N. J.

The effectiveness of nicotinaldehyde thiosemicarbazone in the treatment of hematogenous murine tuberculosis was recently reported by Levaditi *et al.*(1). Independently, this compound was synthesized by Gardner *et al.*(2) and studied by Grunberg and Leiwant(3) in the Roche Research Laboratories. The latter workers, in addition to noting the high degree of effectiveness of nicotinaldehyde thiosemicarbazone in overcoming tuberculosis of mice when infected by the intravenous route, also observed a high degree of activity of this compound in intranasally infected animals. This is of considerable importance because the latter technic of infection leads to the generally more resistant bronchogenic form of the disease.

In contrast to the insoluble *p*-acetylaminobenzaldehyde thiosemicarbazone (Tibione), the nicotinaldehyde molecule is basic in reaction and forms a hydrochloride which is readily soluble. Because of this characteristic, it was considered desirable to determine its degree of absorption in comparison with Tibione. Whereas Tibione may be measured in biological fluids by the Bratton-Marshall technic(4), nicotinaldehyde thiosemicarbazone cannot be coupled with diazonium salts. Therefore a spectrophotometric method, using hexanol for extraction, suggested by Burke and Titus(5) as an alternative technic for measuring Tibione appeared to offer the desired specificity, speed and precision. The method is based on the original work by Spinks(6), who used chloroform. The chemical formulae of these compounds are as follows:


Nicotinaldehyde thiosemicarbazone hydrochloride


p-acetylaminobenzaldehyde thiosemicarbazone (Tibione)

The determination is based on the measurement of ultraviolet absorption maxima of the bands; for nicotinaldehyde thiosemicarbazone at 321 mμ, $E^{1\%}_{1\ \mathrm{cm}} = 1570$ and for Tibione at 331 mμ, $E^{1\%}_{1\ \mathrm{cm}} = 1810$. The ratio $E(320\ \mathrm{m}\mu)/E(342\ \mathrm{m}\mu) = 1.05$ for Tibione.

Procedure. Two ml of heparinized whole blood were added to 2 ml of distilled water in a glass stoppered centrifuge tube. To this was added approximately 350 mg of powdered K_2HPO_4 and 5 ml of normal hexanol. The mixture was mechanically shaken for 10 minutes and then centrifuged at 2,000 rpm for 5 minutes. The supernatant layer of hexanol was then withdrawn and the ultraviolet absorption was measured in a Beckman quartz spectrophotometer directly or after suitable dilution. In all but a few isolated runs, the measured extinction was between 0.2 and 0.8. Blanks were prepared in an

TABLE I. Percent Recovery of Thiosemicarbazones in Hexanol Extraction of Blood.

Blood conc. (γ/ml)	1	3	5	6	8	10	15	25	250
% recovery									
Nicotinaldehyde thiosemicarbazone	93	75	71	73	64	60	72	64	67
Tibione	137	92	88	89	93	92	98	88	—

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1. Levaditi, C., Girard, A., Vaisman, A., and Ray, A., *Compt. rend.*, 1950, v231, 1174.
2. Gardner, T. S., Smith, F. A., Wenis, E., and Lee, J., *J. Org. Chem.*, 1951, v16, 1121.
3. Grunberg, E. and Leiwant, B., Proc. Soc. Exp. Biol. and Med., 1951, v77, 47.
4. Bratton, A. C. and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, v128, 537.
5. Burke, J. C., and Titus, E., Minutes 9th Vet. Admin. Streptomycin Conf., St. Louis, Mo., 1950.
6. Spinks, A., *Brit. J. Pharmacol.*, 1949, v4, 254.

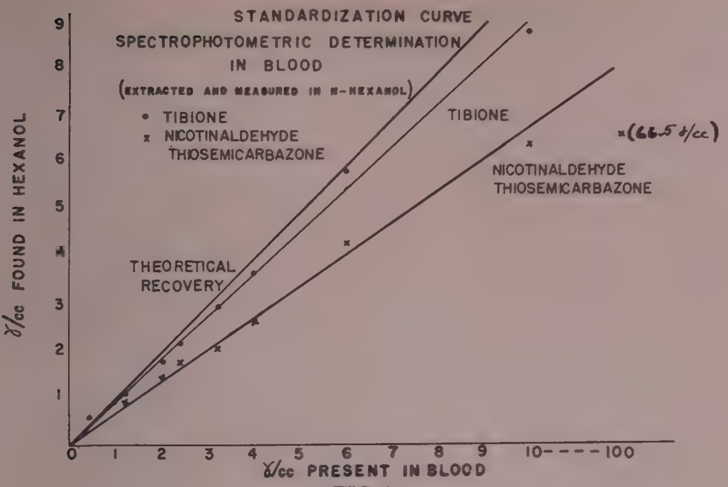

FIG. 1.

identical manner from blood free of the drugs. These were subtracted from the test readings.

Standardization. For the purpose of standardization, 2 ml of normal blood were added to 2 ml of aqueous solutions of the thiosemicarbazones ranging in concentration from 0.1 to 2.5 mg per 100 ml. Nicotinaldehyde thiosemicarbazone was also run at 25 mg/100 ml. These mixtures were then treated as described above. The results are summarized in Fig. 1 and the recoveries in Table I. All solutions were measured against blanks. For dog blood the blanks were found to be: at 321/2 mμ (4 solutions) *avg .067* ± .009 (2 S.D. = *.017*); at 331 mμ (4 solutions) *avg .054* ± .009 (2 S.D. = *.017*). For rat blood the blank was very low (.005). The best range for the analysis (largest slope of per cent absorption vs. concentration) was found to be:

Sample	Hexanol, γ per ml	Corresponding conc (γ per ml) in blood
Nicotinaldehyde thiosemicarbazone	2 -7.5	5-20
Tibione	1.2-5	3-12

The average recovery factors are as follows:

Nicotinaldehyde thiosemicarbazone	.68 ± .036 (Stand. error P=.05)
Tibione	.91 ± .026 (Stand. error P=.05)

These factors were calculated leaving out the value found in the first experiment, where the concentration of the drugs in blood was below the sensitive range.

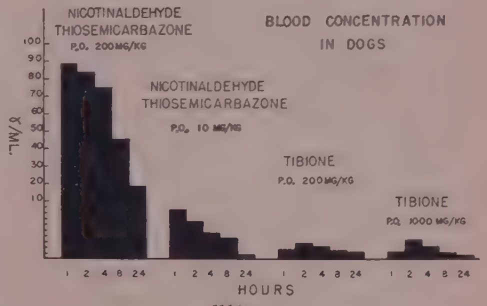

FIG. 2.

The precision of this measurement is estimated to be about 5%, including the uncertainty of the blank. The error is considerably larger outside the best range indicated. This can be avoided easily for higher concentrations by using a suitable dilution but not in the case of too low concentrations without a substantial change in the analytical technic. Beer's law is followed in the range investigated.

Blood concentration in dogs. Nicotinaldehyde thiosemicarbazone and Tibione were administered in single oral doses to dogs. At varying time intervals following dosage, blood was obtained by jugular venipuncture. The results of the spectrophotometric determinations on these specimens are presented in Fig. 2. The highest concentration of Tibione is still below the limit of sensitive determina-

TABLE II. Concentration in Hexanol Extracts of Blood of Rats on Feeding Experiment.

	Concentration in diet:							
	Nicotinaldehyde thiosemicarbazone				Tibione			
	.005%		.05%		.05%		.5%	
	No.	γ/ml found	No.	γ/ml found	No.	γ/ml found	No.	γ/ml found
	1	.19	11	3.40	21	.079	32	.13
	3	.12	12	4	22	.48	33	.84
	4	.076	14	1.55	23	.22	34	1.58
	5	.24	15	3.30	24	.31	35	2.23
	6	.18	16	2.60	25	.44	36	2.48
Avg in hexanol		.16		3		.31		1.45
blood		.4		7.5		.77		3.6
2 stand. error		± .14 (35%)		± 1.4 (19%)		± .037 (48%)		± 2.2(60%)

tion; consequently the limits of error of these measurements are relatively wide, *i. e.*, of the order of 30%. Since the difference between the blood levels achieved is so great, the large error in the determination of Tibione does not influence the comparison.

With nicotinaldehyde thiosemicarbazone (200 mg/kg P.O., Fig. 2) only the last point at 24 hours carries a large error; the 8-hour point has an error of about 10%. In the dog receiving 10 mg/kg the 8 and 24-hour points have larger errors, particularly the latter, so that the 24-hour values in Dogs 2, 3 and 4 are not significantly different. The apparent lack of proportionality between the blood levels in the dogs receiving 200 and 10 mg/kg may be significant, but this conclusion can be only tentative since the differences between the ratios in the two series are at present too close to the estimated limits of error. On the other hand, as judged from the series of standard, the reproducibility of the technic appears to be good, providing measurements are made in the sensitive range.

Blood concentration in rats. Nicotinaldehyde thiosemicarbazone and Tibione, incorporated in ground diet, were administered to weanling, male Sprague-Dawley rats for a period of 13 weeks. At the end of this time, the animals were sacrificed by decapitation and blood was drawn from 5 of each group of 10 animals receiving the drug. The dosage levels were 0.005 and 0.05% of nicotinaldehyde thiosemicarbazone and 0.05 and 0.5% of Tibione. The values, presented in Table II, vary widely. This may be due in large part to differences in the feeding habits of the individual animals.

Discussion. Estimations of p-acetylaminobenzaldehyde thiosemicarbazone have been carried out by various workers using different technics. A colorimetric method using Grote's reagent ($Na_2[Fe(CN)_5H_2O]$) has been described by Mingoja and Moscovici(7). The same authors described an iodometric titration method. Haugas and Mitchell(8) reported a gravimetric procedure based upon precipitation of the thiosemicarbazone by silver nitrate. Colorimetric procedures based on the diazotization and coupling technic of Bratton and Marshall(4) were described by Moss(9) and by Heilmeyer and Heilmeyer(10).

As can be expected from their structures, the recovery of nicotinaldehyde thiosemicarbazone was less favorable than that of Tibione. The recoveries were 68% and 91% respectively.

The present data indicate that nicotinaldehyde thiosemicarbazone hydrochloride, was absorbed to a much greater extent by the animal body than Tibione. The low solubility in body fluids of the latter compound had also been demonstrated by Francis, Spinks and Stewart(11). These workers noted a maximum average blood concentration of 0.37 mg per 100 ml (3.7 γ/ml) of blood with single oral doses of 500 mg/kg to dogs. In

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7. Mingoja, Q., and Moscovici, R., *Arq. de biol.*, 1950, v34, 128.

8. Haugas, E. A., and Mitchell, B. W., *J. Pharmacy and Pharmacol.*, 1950, v11, 759.

9. Moss, D. G., *Lancet*, 1950, v2, 570.

10. Heilmeyer, L., and Heilmeyer, L., *Arch. f. exp. Path. u. Pharmak.*, 1950, v210, 424.

11. Francis, J., Spinks, A. and Stewart, G. T., *Brit. J. Pharmacol.*, 1950, v5, 549.

one of our animals, Dog 3, which received 1,000 mg/kg of Tibione, a maximum blood concentration of 0.33 mg per 100 ml (3.3 γ/ml) was found. This level was even less than that achieved by 1/100 the dose of nicotinaldehyde thiosemicarbazone, *i.e.*, 10 mg/kg. At the end of eight hours after the latter dosage, the level still remained above the maximum level obtained with Tibione. (Fig. 2).

Our results are substantially in agreement with those of a recent work by Spinks(12), who found blood levels of Tibione of the order of 3 γ/ml in the first one to two hours in therapeutic applications. His value (.4 γ/ml) in dogs using a dose of 5 mg/kg is the same as our value for nicotinaldehyde thiosemicarbazone suggesting that at this dosage level the low solubility of Tibione does not yet limit absorption.

The photostability of the compounds in n-hexanol in diffuse daylight can be expected from Spinks' data and appears confirmed by our results. No erratic readings were observed with our standards and all spectrophotometric readings, which were routinely checked back after the absorption curves were recorded, were stable within .005 of the density scale of the Beckmann spectrophotometer. Our recovery factor for Tibione using n-hexanol is .91 as compared to about .80 using chloroform reported by Spinks.

In rats having received comparable amounts of nicotinaldehyde thiosemicarbazone hydrochloride and Tibione, *i.e.*, 0.05% in their diets for 13 weeks, the concentration in the blood of the former agent (7.5 γ/ml) was roughly 10 ten times that of the latter agent (0.77 γ/ml). It is thus apparent from these studies in rats as well as those in dogs that nicotinaldehyde thiosemicarbazone hydrochloride is absorbed to a very great degree as compared with Tibione.

Summary. (1) A spectrophotometric method has been developed for the determination of nicotinaldehyde thiosemicarbazone in blood. (2) Following single oral doses of 200 mg/kg in dogs, the maximum blood concentration achieved by nicotinaldehyde thiosemicarbazone was ten to forty times greater than that of p-acetylaminobenzaldehyde thiosemicarbazone. (3) Following the chronic daily oral ingestion of 0.05% of these drugs in the diet of rats, the average blood concentration attained by nicotinaldehyde thiosemicarbazone was approximately 10 times that attained by p-acetylaminobenzaldehyde thiosemicarbazone.

We wish to acknowledge the technical assistance of Miss Margaret Roe, Mr. Paul Stefko and Mr. Samuel Traiman.

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Changes in Plasma and Tissue Water and Electrolytes After Epinephrine Administration in Rats. (19092)

ABRAHAM DURY AND THOMAS N. JOHNSTON.
From the Dorn Laboratory for Medical Research, Bradford Hospital, Bradford, Pa.

The great difference in the concentrations of potassium in the extracellular and intracellular fluid compartments has often raised the question of hormonal regulation of the "balance" between these two compartments. The adrenal cortex has been particularly considered as the secretory source of hormonal regulation of potassium balance in the organism because of the changes that occur in adrenocortical deficiency and when desoxycorticosterone is used. However, this view has not been generally acceptable(1). The effect of desoxycorticosterone on the renal excretion of potassium has been shown by Harrison and Darrow(2) to be sufficient to explain the change in potassium balance. It is of interest

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1. Gaunt, R., Birnie, J. H., and Eversole, W. J., *Physiol. Revs.*, 1949, v27, 4.

2. Harrison, H. E., and Darrow, D. C., *Am. J. Physiol.*, 1939, v125, 631.

that the relevant literature provides evidence that other known hormonal agents than adrenocortical steroids affected the level of plasma potassium. D'Silva(3), Brewer *et al.* (4), Keys(5) and others have shown that the injection of adrenaline and insulin will cause a fall in the level of plasma potassium in man and animals. Dury *et al.*(6) have reported that there was a distinctive pattern of change in the plasma potassium level in normal humans after the injection of epinephrine which was different from that obtained in epileptic patients and those with Addison's disease. The significance of epinephrine as a hormonal regulator of potassium balance has been raised by the demonstration of Tum Suden(7) that after sympathetic blockade by ergotamine in rats the toxic dose of potassium is diminished, and by the recent report of Dury(8) that adrenalectomized rats were protected from fatal hypergalemic levels when pretreated with epinephrine. Furthermore, Dury(9) has presented evidence that epinephrine decreased the level of plasma potassium without the mediation of the anterior pituitary-adrenocortical axis.

However, no data have been presented in the experiments referred to above on what changes actually occurred in blood and tissue electrolytes after the administration of epinephrine other than its effect upon plasma potassium level. In this report data will be presented on the water and electrolyte content in the blood and tissues of intact and adrenalectomized groups of rats which were untreated compared with those 60 minutes after the administration of epinephrine.

Materials and methods. Normal intact and adrenalectomized (6-7 days) male Wistar rats weighing 225-250 g were used. The rats were given a chow biscuit *ad libitum* and greens twice weekly before they were used in the experiments. The adrenalectomized rats were maintained on 1% NaCl drinking solution. After an overnight fast the animals were anesthetized with n-methylcyclo-hexenyl-methyl barbituric acid (Evipal) and either used immediately without further procedure or were pretreated with a subcutaneous injection of epinephrine and then killed 60 minutes later. The epinephrine (adrenaline tablets; Parke, Davis & Co.) was prepared fresh by solution in physiological saline and injected at a dose level of 0.04 mg/100 g body weight (approximately 0.5 ml). Cardiac blood was obtained in a dry syringe containing 1 drop of heparin (Organon, 10 mg/ml) and immediately centrifuged. Aliquots of plasma were taken for the determination of potassium, sodium, chloride, glucose and the per cent of plasma water. Immediately after the cardiac blood had been obtained the abdomen and thorax were opened; the heart and hepatic vein incised and bled; and a specimen of liver weighing approximately 1 g was removed. The specimen was gently blotted free of blood and weighed immediately. The gastrocnemius muscle of each hind leg was then exposed, freed of nerves lying on its surface or entering the body of the muscle and weighed immediately. One muscle specimen was used for the determination of chloride content by the method of Van Slyke(10). The other muscle and the specimen of liver were used for the analyses of sodium, potassium and water content after weighing, drying and ashing. The specimens were dried overnight at 105°C; and ashed overnight at 425°C. Sodium and potassium determinations of plasma and tissues were done after proper dilutions with the aid of an internal lithium standard flame photometer. Plasma chloride was determined by the method of Schales and Schales(11). Plasma glucose was determined by the photometric method of Kingsley and Reinhold(12). The concentration of plasma electrolytes as

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4. Brewer, G., Larson, P. S., and Schroeder, A. R., *Am. J. Physiol.*, 1939, v126, 708.

5. Keys, A., *Am. J. Physiol.*, 1938, v121, 325.

6. Dury, A., Holler, J. W., and Smith, C., *Fed. Proc.*, 1950, v9, 35.

7. Tum Suden, C., *Am. J. Physiol.*, 1947, v149, 589.

8. Dury, A., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 199.

9. Dury A., *Endocrinology*, in press.

10. Van Slyke, D. D., *J. Biol. Chem.*, 1941, v140, 879.

11. Schales, O., and Schales, S. S., *J. Biol. Chem.*, 1941, v140, 879.

TABLE I. The Reduction in Plasma Potassium 60 Min After Injection of Epinephrine in Intact and Adrenalectomized Rats.

Animals used	% plasma water	Plasma electrolytes m. eq./kg plasma water			Plasma glucose, mg %
		Cl	K	Na	
Normal					
No inj (12)	92.6 ± .06*	108.5 ± .6	4.76 ± .1	158.1 ± .8	102
After epinephrine† (10)	92.4 ± .08	107.6 ± .8	4.41 ± .08 (7) .025‡	156.7 ± .8	301
Adrenx					
No inj (9)	94.1 ± .17	112.8 ± 1.3	5.28 ± .19 (8)	147.7 ± 2.2	45
After epinephrine (13)	93.7 ± .11 (12)	110.8 ± 1.1	4.31 ± .14 (9) .0009	150.8 ± 1.7 (11)	159

* Mean ± S.E. † Epinephrine injected subcutaneously at level of .04 mg/100 g body wt. ‡ P value of the difference between the means occurring by chance alone; when not given P was not significant. No. in parentheses is the No. of rats.

m.eq./kg plasma water and the concentration of muscle and liver electrolytes as m.eq./kg wet tissue were derived from the data. The methods for the calculation of other derived data which are presented in the results were:

$$[\mathrm{Cl}]_e = \frac{[\mathrm{Cl}]_P}{0.95}; \quad \frac{(\mathrm{Cl})_t}{[\mathrm{Cl}]_e} \times 1000 = (\mathrm{H_2O})_e$$

g/kg wet tissue; $(H_2O)_t - (H_2O)_e = (H_2O)_i$ g/kg wet tissue. The subscripts p, t, e and i represent plasma, whole tissue, extracellular and intracellular phase, respectively. The difference between the means of the plasma and tissue water and electrolytes in the groups of untreated and epinephrine pretreated intact and adrenalectomized rats were tested for statistical significance.

Results. Inspection of the data in Table I reveals that aside from the known hyperglycemic effect of epinephrine the level of plasma potassium was significantly decreased in the normal and adrenalectomized groups of rats 60 minutes subsequent to the epinephrine injection. The results indicate that the action of the agent was limited in its effect to the plasma potassium and did not change the concentration of the chloride or sodium nor the per cent of plasma water. The change in the level of plasma potassium was more marked in the adrenalectomized than the intact groups of rats subsequent to the epinephrine injection. A difference in the extent of absolute decrease of the plasma potassium level after epinephrine had been noted in other animal studies(9) of intact and adrenalectomized rats. A similar difference in the extent of plasma potassium decrease after epinephrine has been reported in humans(6). It has been observed that the level of potassium was decreased to a greater extent and over a longer period of time in subjects with Addison's disease than in normal humans after the administration of epinephrine.

The results of the analyses of water and electrolyte content of the gastrocnemius muscle of intact and adrenalectomized groups of rats untreated and after the administration of epinephrine are given in Table II. Inspection of these data reveals that the adrenalectomized group 60 minutes subsequent to the epinephrine had a significant decrease in its potassium content as well as potassium concentration per kg of tissue water in comparison with the control group. There also were significant changes in the distribution of the tissue water with a significant decrease in the intracellular phase concomitant with an increase in the extracellular phase. The change in the muscle water balance is in accord with the change in the potassium content of the tissue. It is noteworthy that the sodium and chloride content of the muscle in the adrenalectomized-epinephrine treated group were not changed when compared with the control group.

In the intact groups of rats epinephrine administration resulted in changes in muscle potassium content and the balance between extracellular and intracellular water similar

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12. Kingsley, G. R., and Reinhold, J. G., *J. Lab. and Clin. Med.*, 1949, v34, 713.

TABLE II. The Effect of Epinephrine on Muscle Water and Electrolytes in Intact and Adrenalectomized Rats.

Animals used	Muscle water, g/kg wet tissue			Muscle electrolytes: m. eq./kg wet tissue			Muscle electrolytes: m. eq./kg tissue water	
	Total	ECW	ICW	Cl	K	Na	K	Na
Normal								
No inj (12)	757 ± 1.2*	92 ± 1.2	664 ± 1.6	10.6 ± .14	99.3 ± 1.6	30.9 ± 1.3	131.1 ± 2.2	40.8 ± 1.7
After epinephrine† (10)	763 ± 1.6	108 ± 3.2	655 ± 3.2	12.2 ± .3	93 ± 1.8	37.2 ± 1.5	121.9 ± 2.5	48.1 ± 1.9
	.006‡	.0001	.01	.0001	.02	.003	.01	.009
Adrenx								
No inj (9)	770 ± 1.4	117 ± 4	653 ± 4.7	13.7 ± .6	90.1 ± 1.6	36.8 ± 1.9	117 ± 2.2	56.4 ± 4.9
After epinephrine (13)	775 ± 1.1	129 ± 3.6	646 ± 3.4	14.9 ± .4	85 ± 1.1	40.8 ± .8	109 ± 1.5	51.5 ± 2.3
	.01	.0001	.0001		.01		.01	

* Mean ± S.E. † Epinephrine injected subcutaneously at level of .04 mg/100 g body wt. ‡ P value of the difference between the means occurring by chance alone; when not given P was not significant.

No. in parentheses is the No. of rats.

to those described in the adrenalectomized group. However, in addition to the above changes there was also a significant increase in both the sodium and chloride content of the muscle. In conjunction with the latter changes it will be noted that the epinephrine-treated intact group experienced a much greater increase in the total and extracellular water content than the adrenalectomized group subjected to the same procedures.

The results of the analyses of liver sodium, potassium and total water content in intact and adrenalectomized groups of rats are shown in Table III. Comparison of the respective epinephrine-treated groups with their controls shows that the total water, sodium and potassium content were not statistically different 60 minutes after epinephrine. It is well known that the hyperglycemia which follows the administration of epinephrine is the result of a rapid hepatic glycogenolysis. It is also known that there is a relationship between liver carbohydrate and potassium metabolism(13,14). Theoretically, therefore, livers which are metabolically in the status of high glycogenolytic activity should be lower than "normally" in potassium content. The results presented here show that this was not the case at the time after epinephrine injection which was chosen for liver analyses. Further investigation of this is certainly indicated.

Discussion. The present results illustrate that epinephrine administered to rats resulted 60 minutes later in a significant decrease in the level of plasma potassium and the potassium content of muscle tissue. There also was a significant decrease in the muscle content of intracellular water together with a significant increase in the extracellular water content. The physiological importance of this mechanism was recently shown(8) by demonstrating that epinephrine protected adrenalectomized rats from an experimentally induced fatal hyperkalemia.

Inspection of Table II showed that the muscle potassium content was decreased 60 minutes after epinephrine administration in

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13. Fenn, W. O., *Physiol. Revs.*, 1940, v20, 377.
14. Fenn, W. O., *J. Biol. Chem.*, 1939, v128, 297.

TABLE III. The Lack of an Effect of Epinephrine on Liver Water and Electrolytes in Intact and Adrenalectomized Rats.

Animals used	Total water, g/kg tissue	Electrolytes: m. eq./kg wet tissue K	Electrolytes: m. eq./kg wet tissue Na	Electrolytes: m. eq./kg tissue H_2O K	Electrolytes: m. eq./kg tissue H_2O Na
Normal					
No inj (12)	686 ± 2.8*	83.7 ± 2 (11)	34.5 ± 2 (11)	122.1 ± 3	50.3 ± 2.8
After epinephrine† (10)	689 ± 2.8	87.7 ± 2.3	33.2 ± 1.3	127.3 ± 3.4	48.1 ± 1.9
Adrenx					
No inj (9)	719 ± 4.1	77.3 ± 3.3	40.5 ± 3.1	107 ± 4.8	56.4 ± 4.9
After epinephrine (13)	720 ± 1.9	83.9 ± 2.2	37.1 ± 1.6	116 ± 3	51.5 ± 2.3

* Mean ± S.E. † Epinephrine injected subcutaneously at level of .04 mg/100 g body wt.
No. in parentheses is the No. of rats.

both the adrenalectomized and intact groups when compared with their respective controls. However, the sodium content of the muscle of adrenalectomized rats was not changed after epinephrine, but both the sodium and chloride content was increased in the muscle of the intact-treated group. It is apparent that some factor(s) other than epinephrine, *per se,* was related to this difference in results between the intact and adrenalectomized groups. There is sufficient evidence in the literature that adrenocortical steroids induce sodium retention in the blood and tissues. It has also been demonstrated that epinephrine will induce the release of adrenal steroids shortly after its injection in an animal. It is therefore suggested that the change in the muscle content of sodium in the intact epinephrine-treated group as distinguished from the absence of any sodium change in the adrenalectomized group was the resultant of the mechanism outlined above. However, the decrease in the potassium content of the muscle tissue of both intact and adrenalectomized epinephrine-treated groups, as well as the significant decreases in the level of plasma potassium, suggest that epinephrine probably has a significant role in the physiological regulation of potassium balance in the fluid compartments of the body.

Summary. Analyses of plasma and tissue water and electrolytes content were made in intact and adrenalectomized groups of rats untreated and 60 minutes after epinephrine injection. Evidence was presented that the plasma potassium concentration was significantly decreased in intact and adrenalectomized rats after epinephrine compared to their respective controls. The potassium content of muscle tissue was significantly decreased in the epinephrine treated intact and adrenalectomized groups. There was no change in the water, sodium or chloride concentration of plasma after epinephrine in either intact or adrenalectomized groups. However, there was a significant decrease in intracellular water content and increase in extracellular water content which was briefly discussed as linked to the change in potassium content. None of these changes were determined in liver tissue of the intact or adrenalectomized groups 60 minutes subsequent to epinephrine injection. In view of the above results and the evidence that the sodium and chloride content of muscle of the intact group was significantly increased after epinephrine but not so in the adrenalectomized group, the results were briefly discussed as evidence of an effect of epinephrine on the potassium "balance" in the compartments of the organism.

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Effect of Varied CO_2 Concentrations of Respired Air on the EEG of the Cat. (19093)

J. G. Pirsch and W. R. McCrum. (Introduced by W. R. Ingram.)

*From the Department of Anatomy, State University of Iowa, Iowa City, Iowa.**

The use of curarizing drugs has been widely accepted as a method for preparing the cat for electroencephalography. Their paralyzing action permits the recording of brain potentials with a minimum of artifact due to muscle activity. This procedure, however, necessitates the use of artificial respiration which introduces the possibility of changes in the gaseous content of the respired air due to the source of the air and rebreathing through the apparatus being used. This brings forth the question of effects produced on the electroencephalogram due to changes in gaseous content of the inspired air. Lennox, Gibbs and Gibbs(1) noted that increase in carbon dioxide tension of blood produced an increase in cortical frequency in the human subject. This observation was furthered by Gibbs, Williams and Gibbs(2) when they correlated decrease in carbon dioxide tension with a decreased frequency pattern in the human electroencephalogram. The problem then is to determine whether such changes occur in the EEG of the experimental animal and whether the changes in the gas, tensions brought about by artificial respiration are of sufficient magnitude to produce these cortical changes. This experiment is an attempt to demonstrate these relationships in the cat.

Materials and methods. Nine normal cats were used for this study, and to decrease resistance and artifact effects on the recordings, a strip of the calvarium, one half inch wide and extending from the frontal sinus to the tentorium cerebelli, was aseptically removed from each. A suitable period was allowed for recovery and healing and electroencephalograms were then recorded. During the recording the animals were paralyzed first with 15 mg of dihydro-beta-erythroidine and then maintained in the paralytic state by a constant intravenous drip of the drug. The animals were maintained on artificial respiration by compressed air and gas mixture delivered through a device which regulated the frequency, pressure and volume of respiration. A valve system (constructed by Dr. M. D. Wheatley) was interposed between the air hose from the respirator and the tracheal cannula. The valves largely prevented rebreathing due to the apparatus. A continuous electroencephalogram was recorded and known mixtures of O_2 and CO_2 were substituted for the normal air supply, 10 minutes for each mixture, at intervals throughout the experiment. Recovery time on normal air was allowed between each mixture. The records were made with an Offner crystograph recorded from 3 monopolar leads, frontal, central and occipital, pasted to the scalp. The reference lead was attached to the ear. Samples of the record for each respiratory phase were analyzed for frequencies by actual measurement of the percentage of fast waves (12-25/sec.), intermediate (8-12/sec.), S_1 slow (4-8/sec.) and S_2 slow (0-4/sec.).

Results. The results of the frequency analysis of these animals are tabulated in Table I. There appears to be very little change in the frequency spectra with the various concentrations of gas that were used. A possible exception to this is the amount of fast waves resulting from the use of a mixture of 95% oxygen and 5% carbon dioxide; however, this difference may not be significant.

Discussion. The results of this experiment indicate that the use of artificial respiration in curarized cats when rebreathing is largely prevented would not seriously alter the EEG

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* This work was aided by a grant from the Central Scientific Fund of the College of Medicine, State University of Iowa.

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TABLE I. Frequency Analysis of the Electroencephalograms With Various Gas Tensions.

Conditions	Frequencies in % F waves, 12-25/sec	I waves, 8-12/sec	S_1 waves, 4-8/sec	S_2 waves, 0-4/sec
Normal air without valve	26	20	41	10
" " with "	35	19	35	8
100% O_2	35	24	34	8
95% O_2 and 5% CO_2	43	19	29	4
93% 7%	30	18	43	5
90% 10%	23	16	49	12

by introducing changes in carbon dioxide tension. If the apparent change in frequency introduced with the 5% carbon dioxide is significant, a safe margin is, nevertheless, present in the use of a normal air supply which contains about a 0.04% concentration of that gas. It must also be recalled that the amount of oxygen administered with the carbon dioxide is many times that found in normal air. Alterations in gaseous content due to air supply and equipment would, therefore, be slight in contrast to proportions used in this experiment, and cortical effects would be of minimal values.

Summary. In cats curarized with dihydrobeta erythroidin hydrobromide and under artificial respiration, changing from air to mixtures of 3 to 10% CO_2 in oxygen caused no significant change in the percentages of fast, intermediate and slow waves in the electroencephalographic spectrum. It is concluded that artificial respiration as used in experiments with curarizing drugs does not distort the frequency spectrum.

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Blood Sugar Levels and Cataract in Alloxan-Treated, Galactose-Fed and Xylose-Fed Weanling Rats.* (19094)

REX E. STERLING† AND PAUL L. DAY.

From the Department of Biochemistry, School of Medicine, University of Arkansas, Little Rock.

Cataract has been produced experimentally in the rat by a number of means. Among them are riboflavin deficiency(1); tryptophan deficiency(2); the feeding of lactose (3), galactose(4), or xylose(5); and treatment with alloxan(6). The cataract of riboflavin deficiency may be distinguished with the aid of the ophthalmoscope from that seen in tryptophan deficiency, and each of these may be distinguished from the cataract resulting from the feeding of lactose, galactose, or xylose. The cataracts resulting from lactose-, galactose-, or xylose-feeding, however, are ophthalmoscopically indistinguishable. Darby and Day(5) found that those carbohydrates were cataractogenic which elevated

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[†] United States Public Health Service Predoctorate Research Fellow.

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TABLE I. Average Time of Appearance of Cataract, Incidence of Cataract, and Average Blood Sugar Levels in Groups of Weanling Rats.

		Appearance of cataracts				Blood sugar	
		Incipient cataract		Gross cataract			
Group	No. of rats	Avg time, days	Incidence, %	Avg time, days	Incidence, %	No. of determinations	Level, mg/100 ml
I. Control	10	—	0	—	0	70	117.8 ± .4[*]
II. Galactose-fed	10	4	100	20	90	80	244.5 ± 3.9
III. Xylose-fed	9	3	100	7.5	90	77	301 ± 32.1
IV. Alloxan diabetics (150 mg/kg)[†]	9	17.6	77.8	—	0	82	292 ± 88
V. Alloxan diabetics (200-250 mg/kg)[†]	7	9.6	100	56	30	43	522.2 ± 82.9

[*] Stand. error of the mean. [†] Size of single injected dose of alloxan.

the blood sugar levels of weanling rats when fed as 35% of the diet; carbohydrates which did not raise the blood sugar level were not cataractogenic. It was not clear from that study, however, whether it was the blood *level* of sugar or the *specific configuration* of the sugar concerned which was responsible for the cataract production.

By the use of rats made diabetic with alloxan it has been possible to compare the cataractogenic action of a high level of blood glucose with similar levels of blood sugar in galactose- and xylose-fed littermates.

Experimental. Preliminary experiments indicated that diabetes could be produced in the weanling rat by a single injected dose of alloxan of from 150 to 250 mg per kilo of body weight. Weanling albino rats of both sexes were divided into groups of 10 or more animals each, matched as to weight, sex, and litter. They were given diets having the following percentage composition: Labco casein 18, salt mixture(7) 2, cod liver oil 2, vegetable shortening 6, carbohydrate 72, and a suitable vitamin mixture(8). The control group and the alloxan-treated groups received the above diet with D-glucose as the only carbohydrate. Other groups were given similar diets containing 35% of D-galactose and D-xylose respectively, replacing a like amount of D-glucose; these groups are hereafter referred to as galactose-fed and xylose-fed. The eyes of each animal were examined with an ophthalmoscope daily during the early part of the experiment and at weekly intervals thereafter. The pupils were dilated with atropine sulfate prior to such examinations. Blood sugar analyses were made at weekly intervals or oftener.

Results and discussion. Table I gives a summary of the important data. The term "incipient cataract" refers to the first evidence of lens opacity as seen with the ophthalmoscope. This was usually evident by the appearance of bubbles at the periphery of the lens. The term "gross cataract" refers to the stage of opacity in which the eye appeared milky; an ophthalmoscope was not required to recognize this condition.

The untreated controls receiving D-glucose showed no cataract and their blood sugar levels were in the normal range. Rats receiving either galactose or xylose all developed cataract, and blood sugar levels were greatly elevated. Most of the alloxan-treated rats developed some lens opacities, although in only a few of them did the condition proceed to the condition referred to as gross cataract. The xylose-fed rats showed lens opacities earliest, yet the group of rats given the larger dose of alloxan (200-250 mg/kg) exhibited the highest average blood sugar level. It thus appears evident that there was no simple relation between the average blood sugar level and the rapidity with which cataract developed. If it is assumed that the cataract resulted from elevated blood sugar levels, then it is evident that a sufficiently high level of glucose can be thus injurious to lens tissue. It would appear, however, that either

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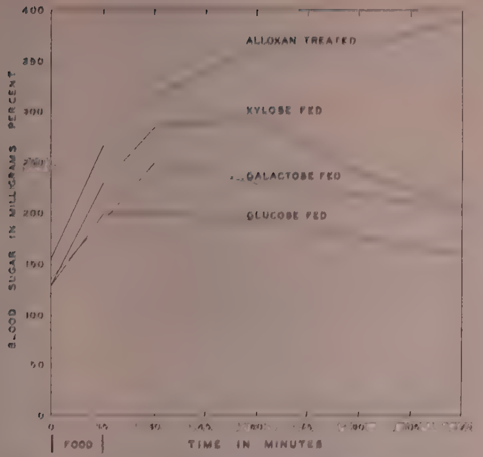

FIG. 1. Tolerance curves of individual rats from each group. Food was withheld for 12 hr. Blood was drawn (zero time), the animals were allowed access to their respective diets for 15 min, the food was again withheld, and blood samples were taken for analysis as shown.

galactose or xylose is injurious to the lens at a lower blood level.

Fig. 1 gives blood sugar tolerance curves for typical rats from each group.

Summary. Cataract was produced experimentally in weanling littermate rats by (1) alloxan treatment, (2) galactose feeding, and (3) xylose feeding. All groups showed greatly elevated blood sugar levels as compared with untreated controls receiving glucose. Cataract was exhibited by all of the xylose- and galactose-fed rats, and by nearly all of the alloxan-treated rats. However, the development of cataract was much slower in the alloxan-treated than in the other groups. It is concluded that not only the level of blood sugar, but also the specific configuration of the sugar concerned is a factor in its cataractogenic action.

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Some Pharmacological Properties of Three New Mercurial Diuretics.*

(19095)

Carroll A. Handley, Don Chapman, and John H. Moyer.

From the Departments of Pharmacology and Medicine, Baylor University College of Medicine, Houston, Texas.

A preliminary appraisal of a new series of mercurial compounds with diuretic activity showed many in the group to have greater potency than diuretics currently employed(1). From stability data and other considerations, 3 of the agents were selected for further investigation. They are 3-chloromercuri-2-methoxypropylurea (1347Ex), 3-carboxymethyl-mercaptomercuri- 2 -methoxypropylurea (1353Ex), and 3-(α-carboxyethylmercaptomercuri)-2-methoxypropylurea (1431-Ex). The following data compare some of the pharmacological properties of these mercurials with meralluride (Mercuhydrin).

Methods. Diuretic activity and sodium excretion were studied in unanesthetized female dogs or in dogs lightly anesthetized with sodium pentobarbital (25 mg/kg, I.V.). The animals had not had access to food for 16-18 hours and water for 3-4 hours prior to the experiment. Urine, collected at 20 minute intervals from a retention catheter, was removed from the bladder as completely as possible by introducing air and using compression. Three or 4 control collections were made before the drugs were administered. Meralluride at several dosage levels was used as a standard, and the dosage of the other compounds was adjusted to give approximately equivalent responses. A Perkin-Elmer flame photometer was used for sodium analyses. Electrocardiograms, in dogs anesthetized with 30 mg per kg of pentobarbital sodium, recorded from lead II, were used to investigate the acute cardiac effects. After a control record, amounts of the 3 new mer-

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* Aided by a grant from Lakeside Laboratories, Milwaukee, Wisc.

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curials equivalent to 250 mg of mercury dissolved in 50 ml of physiological saline were infused at a rate of 2 ml per minute into the external jugular vein. Electrocardiograms were taken at 5 minute intervals for the first hour, and then at half hour intervals for an additional 2-3 hours. In the chronic toxicity studies, changes in glomerular filtration rate (GFR) was used as a criterion for the development of renal damage, and the animals were carefully observed for other symptoms of mercury poisoning. In addition, the maximal rate of tubular reabsorption of dextrose (TmG) was used as a measure of tubular function in 7 and 3 dogs given 1347Ex and 1431Ex, respectively. The method of Lang and Nelson(2) was used for the determination of mercury in urine and feces.

Diuretic potency. Ten dogs were used for measuring the activity of each compound at each dosage level. All the assays of diuretic activity were made from a group of 20 animals of 12-16 kg. These animals were used for each mercurial, although none of the dogs was given a diuretic more frequently than once a week. No difference could be detected in the diuretic response between unanesthetized animals and those lightly anesthetized with sodium pentobarbital. The average control rate of urine formation in 10 unanesthetized dogs was 0.11 ml/min and the maximum rate after 4 mg/kg of meralluride mercury intravenously was 3.10 ml/min. Tests on the same animals several days later under the same conditions except that they were lightly anesthetized with 25 mg/kg of sodium pentobarbital intravenously gave corresponding figures of 0.15 and 3.42 ml/min. Diuretic activity of the 3 compounds, tested at different intravenous dosage levels, was 3 or 4 times that of meralluride as measured by the maximum output of urine and sodium per minute. Table I shows the average response of 10 dogs for each dosage. The development of maximal diuresis was usually more delayed than with meralluride, but the duration of action was approximately the same, namely 5 or 6 hours.

TABLE I. Comparison of Average Maximal Rates of Excretion of Urine and Sodium of 10 Dogs for Each Dose.

Compound	Dose, mg Hg/kg	Avg control urine, ml/min	Avg max rate of excretion	
			Sodium, mEq/min	Urine, ml/min
Meralluride	2	.07	.43	2.43
	4	.10	.64	3.10
1353Ex	.5	.12	.32	2.28
	1	.17	.56	3.67
1347Ex	.5	.10	.34	2.12
	1	.14	.43	3.22
1431Ex	.5	.11	.48	2.84
	1	.13	.61	4.29

TABLE II. Recovery of Mercury in Urine and Feces After Intravenous Administration of Doses Equivalent to 2 mg per kg of Mercury.

Compound	Avg of 12 dogs per compound (%)			
	24 hr	48 hr	Total	Range
Meralluride	87	4	91	75-98
Mercaptomerin	65	11	76	52-90
1353Ex	80	3	83	63-96
1347Ex	82	3	85	54-94
1431Ex	88	2	90	49-97

Recovery of mercury. Mercury determinations on 24 and 48 hour specimens of urine and feces showed that most of the mercury, from intravenous doses of the compounds equivalent to 2 mg per kg of mercury, was excreted within 24 hours. The amount of mercury recovered from the feces was usually less than 4% of the total mercury excreted. Table II compares the rates of excretion of the new mercurials with meralluride and mercaptomerin (Thiomerin). The rates of excretion approached that of meralluride and appeared to be somewhat greater than the rate of excretion of mercaptomerin. This difference was especially apparent at the end of 24 hours.

Acute cardiac effects. Six dogs, weighing 10-13 kg, were used for evaluating the cardiac effects of each compound. Five dogs given 1431Ex showed no significant cardiac changes during the infusion period, but one animal developed ventricular fibrillation at the end of 2 hours. None of the dogs receiving 1353Ex showed any evidence of cardiac effects. The QRS interval increased in all dogs infused with 1347Ex, and 2 developed ventricular fibrillation. All animals given

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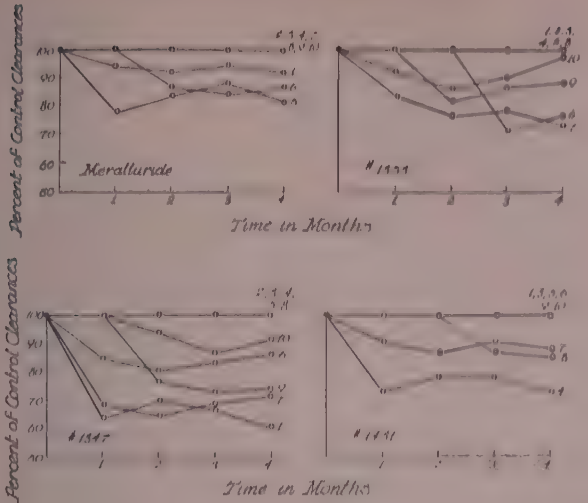

FIG. 1. Comparison of the effect of 3 mercurial diuretics with meralluride on glomerular filtration rate.

these large doses succumbed within 24-72 hours. The usual autopsy findings were pulmonary edema and gross hemorrhage of the gastro-intestinal tract.

Chronic toxicity. Chronic toxicity of the 3 compounds was compared to that of meralluride by determining changes in glomerular filtration rate at monthly intervals. The dogs were frequently examined for the appearance of dermatitis, and other symptoms of mercury poisoning. Ten dogs were used for each drug and were given an intravenous dose equivalent to 1 mg of mercury per kg 3 times weekly, except for the dogs in the meralluride group. Since meralluride is approximately one-fourth as active in producing diuresis in the dog as the other 3 compounds, it was administered in an intravenous dose equivalent to 4 mg of mercury per kg. These doses are several times the therapeutic level as used in man and were selected because they approach the maximum tolerated dose for the dog. Fig. 1 shows the effect of 4 months of such treatment on glomerular filtration rates. Each number in the graphs represents a curve for glomerular filtration rates of one dog determined at monthly intervals. The curves show that with these comparatively large and equivalent diuretic doses, there were no marked differences in renal toxicity among the three new compounds, or between the new agents and meralluride. Mild dermatitis developed in one dog in the meralluride group, 2 in the 1347Ex group, and one receiving 1353Ex. All animals developing dermatitis also showed a reduction in glomerular filtration rates. No diarrhea, stomatitis, or other symptoms of severe mercury poisoning that occur in dogs were noted in any of the animals.

Discussion. The greater diuretic activity of these compounds, considered in relation to the fact that they are excreted at rapid rates, should give them a safety margin equal to, or even greater than, mercurial diuretics currently used. Chronic toxicity data on dogs, receiving relatively large doses over a period of several months, indicate that there is little difference in renal toxicity, or other signs of mercury poisoning, when these agents are compared with an equivalent diuretic dose of meralluride. An extensive study has previously been made to show the changes in renal function associated with the development of chronic toxicity from several mercurial diuretics currently used therapeutically. The effects on glomerular filtration rate have been described(5) and the changes in renal tubular function, as measured by TmG and TmPAH, will soon appear in connection with

TABLE III. Comparison of Effect of Mercurial Diuretics on Glomerular Filtration Rate and Tubular Function.*

Dog No.	Wt, kg	Drug	Control			1-4 mo of mercurial		
			GFR,† ml/min	TmG,† mg/min	GFR/TmG	GFR,† ml/min	TmG,† mg/min	GFR/TmG
M-4	13	Meralluride	45	156	.29	22	87	.25
8	17	”	60	232	.26	45	170	.26
T-5	14	Mercaptomerin	55	207	.27	25	82	.31
7	19	1347Ex	67	240	.28	40	130	.31
1	17	”	83	275	.30	50	164	.30
4	12	1431Ex	53	169	.31	37	128	.29
M-2	15	Meralluride	64	208	.31	61	191	.32
3	17	”	76	273	.28	83	301	.28
1	15	1347Ex	58	215	.27	53	211	.25
2	16	”	54	203	.26	49	185	.27

* Arterial blood samples used for all analyses. Dogs unanesthetized.
† Average of 3 10-min periods.

a study of several types of agents having nephrotoxicity. The investigation included weekly determinations of the above renal functions in 20 dogs given daily injections of diuretics (4 mg/kg of mercury, I.V.) for one month. In addition, similar observations were made on 5 dogs given mercuric chloride (0.2 mg/kg I.V., daily). The data in Table III show representative examples taken from the previous study (M-4, -8, -2, -3 and T-5) and similar observations on 1347Ex and 1431Ex from the present investigation. The significant feature of the renal function studies is to demonstrate parallel changes in GFR and tubular function as may be seen by the constant GFR/TmG ratio. A similar correlation between GFR and TmPAH has been observed as nephrotoxicity develops from mercurial compounds. The logical conclusion to be derived from such data is that variable numbers of nephrons are completely inactivated as renal toxicity develops. Depressed renal functions develop either simultaneously with, but more frequently before, other signs of chronic mercury poisoning. In some animals, GFR and TmG returned to control values several weeks after discontinuing the drugs, while in others the damage appeared to be permanent. The GFR and TmG of dogs M-4 and T-5 in Table III had not improved significantly 2 months after discontinuing the drugs. Mercuric chloride produces similar chronic toxic effects as organic mercurial compounds, including the above mentioned renal changes. The last 4 dogs illustrate the absence of renal effects when no other signs or symptoms of chronic mercury toxicity were demonstrable.

Because of the proportional depression of GFR and tubular functions with the development of chronic toxic effects from mercurial diuretics, we have used GFR alone to indicate the development and progress of renal damage from these agents. Certain precautions must be observed if GFR is to be used as a measure of renal toxicity. Several hours of mercurial diuresis will frequently lower GFR and TmG considerably from dehydration(6). Recovery from extensive water loss is rapid, after the diuretic action of the drug disappears, and may be quickly eliminated by intravenous saline. All the data on renal functions reported were obtained on a day when no drug was given or prior to the administration of the diuretic. Routine hematocrit determinations also served to indicate the presence or absence of hemoconcentration.

Studies in this laboratory have indicated that doses of meralluride equivalent to 8 mg/kg of mercury, given intravenously to dogs, consistently produced electrocardiographic changes. In fact, 4 mg/kg is the minimum dose that may be administered without prolonging the QRS complex in some animals. The cardiac effects appeared

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promptly after a single injection of the above specified amounts of meralluride over a one minute period. Previous observations both in cats and dogs have yielded similar results(3,4).

Dogs tolerated large amounts of 1353Ex and 1431Ex without any indication of cardiac toxicity, which might have been anticipated since these two compounds are mercaptides (3). The chlorine substituted mercurial (1347Ex) appeared to have even less cardiac toxicity than meralluride, which is probably the least toxic of the non-mercaptide mercurials(3,4). No electrocardiographic changes occurred in any of the dogs given 1347Ex until the total amount infused was equivalent to 75 mg/kg of mercury and in 4 dogs, the QRS complex was not prolonged until about twice this amount of the compound had been given. These values are many times the amount of meralluride mercury required to produce similar electrocardiographic changes.

Summary. The compounds, 3-chloromercuri-2-methoxypropylurea (1347Ex), 3-carboxymethylmercaptomercuri - 2 - methoxypropylurea (1353Ex), and 3-(α-carboxyethylmercaptomercuri)- 2 -methoxypropylurea (1431-Ex), had 3 or 4 times the diuretic potency of meralluride in the dog. Twenty-four and 48 hour mercury excretion rates were comparable to that of meralluride at the same dosage level. Chronic toxicity studies indicated no essential difference between meralluride and the other compounds, if the greater diuretic potency of the new compounds was considered and the dosage adjusted accordingly. Large amounts of 1353Ex and 1431Ex were infused intravenously without disturbing cardiac function, but the acute cardiac toxicity of 1347Ex, although greater than the other 2 compounds, appeared to be less than meralluride.

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Distribution of Phosphorus^{32} in the Embryo and Larva of the Frog.* (19096)

Louis A. Hansborough and David Denny. (Introduced by A. H. Maloney.)

From the Department of Zoology, Howard University, Washington, D. C.

Radioactive substances have been employed in several studies on protein synthesis during development. Recently, Friedberg and Eakin (2) studied the incorporation of C^{14} of radioactive glycine into protein in the embryos and larvaė of the Pacific tree-frog, *Hyla regilla.* They reported an increase in the uptake of glycine from the beginning gastrula through the early larval stages. This was interpreted as evidence of acceleration in protein synthesis accompanying differentiation and growth. Dent(1) observed the effects of phosphorus^{32} on regenerating amphibian tissues. He found that tadpoles of *Rana clamitans* immersed for 3 days in solutions of P^{32} (1000 microcuries) before amputation of the tail, regenerated a tail which was half as large as that of the control after 2 weeks. By introducing radioactive phosphorus into the hen's egg and analyzing chemical extracts of the tissues of the embryos, Hevesy, Levi and Rebbe(4) showed that the phosphatide molecule of the yolk was lower in specific activity than that of the embryo and con-

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* This work was supported in part by a special grant from the Atomic Energy Commission under Contract No. AT(30-1)-949.

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cluded that the more active molecule is synthesized in the embryo. In the embryo itself, a metabolic gradient of phosphorus metabolism has been described as early as the 18th hour of incubation(6). Hansborough and Nicholas(3), using radiophosphorus in the chick embryo found that the uptake of P^{32} increases with the age of the embryo and that the most active regions of the embryo are regions where the population of cells is greatest. They observed no tissue specificity in the process.

Since it is more easily observed and its morphological development is well known, it seemed desirable to extend the latter study to the amphibian embryo.

Methods. Male and female frogs *(Rana pipiens)* in amplexus were collected at the Kenilworth Aquatic Gardens, Washington, D. C. Fertilized eggs were obtained from these, divided into 3 groups of 225 each and placed in finger bowls containing 750 ml of radioactive NaH_2PO_4. The solution in the first container had an activity of 550 microcuries: the second. 300 microcuries and the third, 65 microcuries.† The developing eggs were allowed to remain in these solutions until the desired stages were reached. Similar groups were prepared and exposed periodically for 2 hours every 24 hours and then returned to tapwater. The stages were determined according to the stages of development outlined by Rugh(7) after Shumway. The temperature and water level in each container were kept constant. Embryos and larvae were prepared for autoradiographs according to the method of Holt, Cowing and Warren(5). Whole embryos used for autoradiographic study were embedded in paraffin blocks. Those prepared for sectioning were cut at 10 microns and mounted on microscope slides. Triple-X panchromatic sheet film was used in preparing autoradiographs. The film was placed in close contact with whole mounts and sections, wrapped tightly in black paper, bound with cellophane tape and rubber bands and exposed for 5 days. They were then developed in Kodak Selectol (D-52) and fixed according to the usual procedure. For microscopic study, sections were stained in Delafield's hematoxylin and eosin and mounted in permount. All photographs were made with a Leica Microcamera. The magnifications of microphotographs and autoradiographs are indicated under explanation of figures.

Results and conclusions. Embryos exposed to different concentrations of P^{32} show the highest uptake of phosphorus in the solutions of greatest concentration of phosphorus (550 microcuries), which is to be expected. Those in the lowest concentrations showed correspondingly fainter autoradiographs. This is true of all stages of development. Those exposed periodically for 2 hours in every 24 hours and returned to tapwater gave autoradiographs similar to those exposed continuously, except that the blackening on the film was less intense. Hence, variations in the time of exposure did not affect the distribution pattern of the phosphorus.

Since the embryos in the highest concentration of phosphorus gave clear yet typical results, they will be described in detail according to the developmental stages observed.

1. Mid-cleavage (Fig. 1). Autoradiographs show that the peripheral cells are mainly involved in the uptake of P^{32}. This is to be expected since the surface cells are in direct contact with the medium. Both the micromeres at the animal pole and the yolk-laden cells at the antipole show an equal distribution of P^{32}. Cells toward the center of the embryo, however, show only slight activity.

2. Mid-gastrula (Fig. 2). Autoradiographs of the whole embryo show blackening of the same intensity as that of the preceding stage. Those of the sectioned embryo show a concentration of P^{32} not only at the periphery but in the yolk-laden cells of the gut as well. Sections also show a concentration of P^{32} at the blastopore where population of cells is high and cell division is rapid.

3. Neural Tube (Fig. 3). The whole embryo shows less activity than the midgastrula.

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† We are indebted to Dr. Herman Branson of the Departmment of Physics for the preparation of these solutions.

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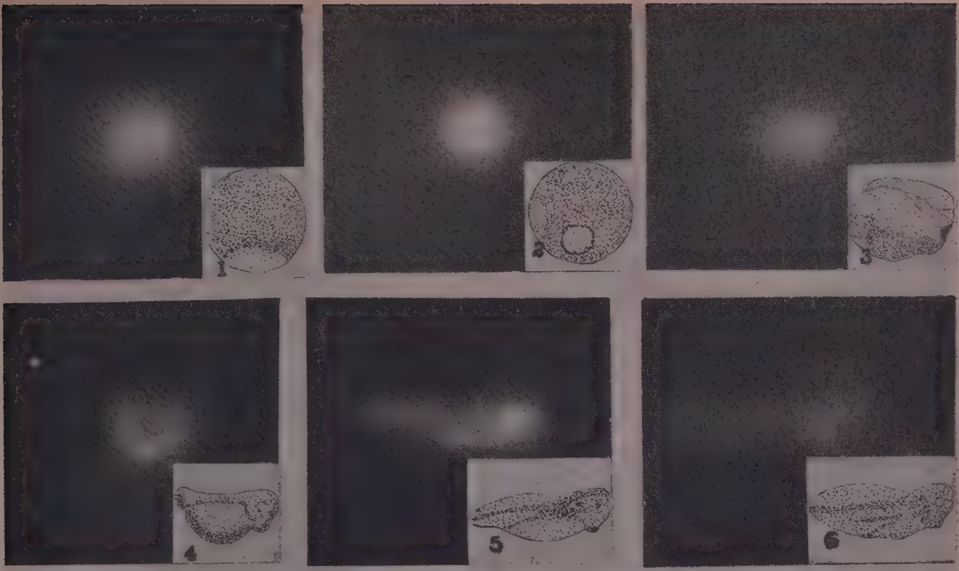

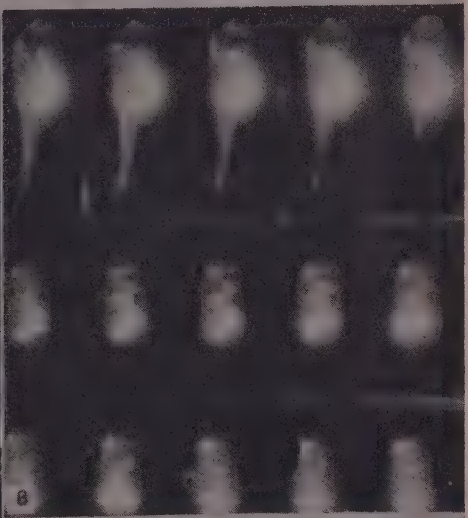

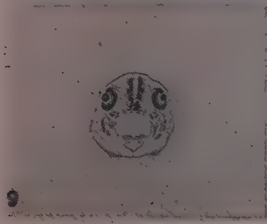

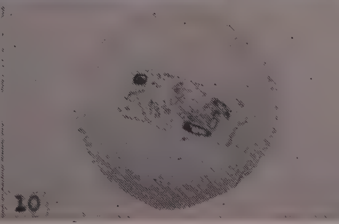

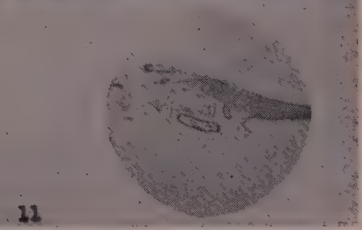

FIG. 1-6. Autoradiographs and drawings of embryos in the (1) mid-cleavage stage, (2) mid-gastrula stage, (3) neural tube stage, (4) muscular response stage, (5) gill circulation stage and (6) tail-fin circulation stage. × 4.

FIG. 7. Autoradiograph of sections of an embryo in the operculum complete stage at the level of the eyes and otocyst. × 4.

FIG. 8. Autoradiograph of longitudinal sections of an embryo in the same stage of development as that shown in Fig. 7. × 4.

FIG. 9. Microphotograph of one of the sections (through the eye) shown in Fig. 7. × 8.

FIG. 10. Microphotograph of one of the sections (middle row) shown in Fig. 8. × 4.8.

FIG. 11. Microphotograph of one of the sections (top row) shown in Fig. 8. × 4.8.

Sections, however, show that the phosphorus is no longer concentrated in the surface epidermis but also in the oral suckers, neural tube, eye, fore-gut and the liver. Mesodermal areas and the ears show less activity.

4. Muscular Response (Fig. 4). The whole embryo shows high activity on the outer surface, particularly in the anterior, posterior and ventral regions. Sections, however, show that there are several internal regions which give a more intense blackening on the photographic film. These regions of high activity include oral suckers and the muscles. Next in activity are surface ectoderm, gut, neural tube, eyes, liver, gills and notochord.

5. Gill Circulation (Fig. 5). Less activity is shown by the whole embryo in this stage than that in stage 18 (see chart), except in the eyes and gut. Sections show that in addition to the eyes and gut, the oral suckers and gills are also regions of high activity. Muscles and notochord show less activity than in the preceding stage, while mesenchyme, lateral mesoderm, heart, notochord and tail are the least active structures.

6. Tail-fin Circulation (Fig. 6). The embryo of this stage appears to be the least active of the series. Sections show that the gut, particularly the fore-gut, is most active. This is probably due in part to the entrance of water containing the phosphorus during respiratory movement. Next in activity are the eyes, ears, kidney tubules, liver, muscles, tail and teeth. The heart, lateral mesoderm and mesenchyme show only slight activity.

7. Operculum Complete (Fig. 7). The greatest activity is observed at this stage. The whole embryo produces intense blackening on the photographic film (not shown). In sections, the following structures show the highest activity: gut, eyes, gills, teeth and cartilage of the head. Other structures of the embryo are only slightly less active than these regions.

A study of the table will show that regions of high activity may show slight shifts in different stages of development. It is difficult to account for such shifts unless we assume that the shift of high activity from a specific region of an embryo in an early stage of development to a different region in a later stage

TABLE I. Distribution of Phosphorus^{32} in Embryos of Different Development Stages.

Developmental stage from Shumway's table	Stage No.	Ecto-derm	Ent. (gut)	Mesen-chyme	Neur. tube	Eye	Ear	Lateral mesod.	Heart	Kidney tubule	Liver	Muscles	Oral sucker	Gill	Notch	Tail fin	Teeth
Mid-cleavage	9	2+	1+														
Mid-gastrula	12	2+	2+														
		Blastopore 3+															
Neural tube	16	2+	2+		2+	2+	1+	1+		1+	2+		2+		2+		
Muscular response	18	2+	2+	1+	2+	2+	1+	1+	1+	1+	2+	3+	3+	2+	2+		
Gill circulation	20	2+	3+	1+	2+	3+	1+	1+	1+	1+	2+	2+	3+	3+	1+	1+	
Tail-fin circulation	22	2+	4+	1+	2+	3+	3+	1+	1+	3+	3+	3+	2+	2+	2+	3+	3+
Operculum complete				Cartilage													
	25	3+	4+	4+	3+	4+	3+	2+	2+	3+	3+	3+	3+	4+	3+	3+	4+

Degree of blackening: 1+, slight; 2+, low; 3+, intermediate; 4+, deepest.

is an indication of a shift in the rate of the utilization of P^{32} in these regions.

It is concluded from a study of the autoradiographs of whole and sectioned embryos that P^{32} is rapidly utilized in those regions where cell duplication is rapid and hence, cell population is high. This is in agreement with the observations on the distribution of P^{32} in the chick embryo (Hansborough and Nicholas)(3). The process is a cumulative one, increasing with the age of the embryo.

Summary. (1) Fertilized eggs of the leopard frog, *Rana pipiens,* were exposed to solutions of radiophosphorus of different activities (65, 300 and 550 microcuries). (2) Autoradiographs and microphotographs were prepared from whole and sectioned embryos in 7 different stages of development. (3) Autoradiographs show that the uptake of phosphorus^{32} is greatest in embryos exposed to the most active solutions and also that the process is a cumulative one, increasing with the age of the embryo. (4) The most active areas of the embryo in each of the stages studied are those areas where cell division is high and the population of cells is largest. (5) With the appearance and differentiation of organ primordia, the rate of phosphorus turnover increases, thus indicating that phosphorus is probably being utilized more rapidly in those areas in the synthesis of nucleoproteins.

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Proposed Intermediary Role of 3,4-Dihydroxyanthranilic Acid in the Biosynthesis of Nicotinic Acid.* (19097)

L. M. HENDERSON, HELEN N. HILL, RUTH E. KOSKI, AND I. M. WEINSTOCK.†
From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana.

The conversion by rat liver slices and homogenates of 3-hydroxyanthranilic acid to quinolinic acid, but not to nicotinic acid has been reported(1,2). Neither tryptophan nor kynurenine gives rise to either of these pyridine derivatives under similar conditions(1). It has been assumed, that in the conversion of 3-hydroxyanthranilate to quinolinate there are at least two enzyme catalyzed steps, since the reaction involves a four electron oxidation and the conversion of a benzene to a pyridine nucleus. The work of Stanier *et al.*(3), and other evidence bearing on the mechanism of metabolic breakdown of aromatic compounds led to the suggestion(4) that 3,4-dihydroxyanthranilic acid might be an intermediate in the postulated 3,4 fission of 3-hydroxyanthranilate. Mitchell *et al.*(4) found that this compound was not active for *Neurospora crassa.* Likewise, a sample kindly supplied by Dr. Mitchell, failed to give rise to quinolinic acid when incubated with rat liver slices. More recently Makino *et al.* (5) have reported that slices of liver from a number of species form nicotinic acid as estimated by the cyanogen-bromide-aniline color reaction, when incubated with tryptophan, 3-hydroxyanthranilate, 3,4-dihydroxyanthranilate, and quinolinate. They used 24-hour incubation periods with beef, hog and horse liver. Since these findings are at variance in several respects with our results with rat liver preparations, we have reinvestigated the

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* Supported in part by a grant from the Nutrition Foundation, Inc., New York.

† Present address. New York Hospital, New York City.

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TABLE I. The Formation of Nicotinic Acid and Quinolinic Acid by Rat Liver Slices.

Substrate		Pyridine derivatives found	
		Nicotinic acid	Quinolinic acid
	3 hr incubation		
		μg per 150 mg	μg per 150 mg
0		9.7	1.4
1 μM	L-tryptophan	11.8	0
10 μM	”	10.7	1.4
1 μM	DL-kynurenine	10.9	1.4
1 μM	3-OH anthranilic acid	11.8	56
1 μM	3,4 di OH anthranilic acid	11	−.5
17 μM	” *	11.9	1.4
1 μM	quinolinic acid	10.2	121
	24 hr incubation		
		μg per g	μg per g
0		144	12
2 mg	L-tryptophan	166	6
3	DL-kynurenine	138	31
—	3-OH anthranilic acid†	—	1000
1.7	3,4- ” ”	136	22
1.6	quinolinic acid	98	1700
	24 hr incubation		
		μg per 2 g	μg per 2 g
0		195	50
2 mg	L-tryptophan	218	46
3	DL-kynurenine	200	27
1.5	3-OH anthranilic acid	200	1040
1.7	3,4-di OH anthranilic acid	195	49
1.6	quinolinic acid	215	890‡

* Added as a dry powder.

† Portion of this sample was lost after incubation. Quinolinic acid value is the amount in remaining sample.

‡ In 4 hr incubation exp. with 150 mg of hog liver slices and homogenates and 5 μM of quinolinic acid, all of the substrate was recovered.

general problem using hog and rat liver slices in long term *in vitro* experiments. In addition, 3,4-dihydroxyanthranilate has been studied *in vivo* with regard to its growth promoting activity for rats and *Xanthomonas pruni* and its effect on quinolinic acid excretion by the rat.

Experimental and results. In vitro studies. 3,4-dihydroxyanthranilic acid was prepared from vanillin by the method outlined by Makino and associates(5). DL-kynurenine and 3-hydroxyanthranilic acid were the samples used in previous studies(6,7). For the *in vitro* studies, approximately 150 mg of fresh liver as slices or homogenate was suspended in a total volume of 3 ml of Krebs-Ringer phosphate buffer in 10 ml Erlenmeyer flasks or 18 x 150 mm test tubes. The substrate was added in 0.2 ml of buffer. In some experiments, 3,4-dihydroxyanthranilic acid was added in a finely powdered form from time to time during the incubation to preclude destruction by autooxidation during the shaking required to dissolve this compound. Control flasks containing inactivated enzyme plus substrate, or active enzyme but no substrate, were included. The reaction mixtures were incubated exposed to the atmosphere at 38°C with shaking for 30 minutes, 3 hours or 24 hours. The reaction was stopped by immersing the vessels in a boiling water bath for 3 minutes or by the addition of 0.5 ml of a 50% solution of trichloracetic acid. Nicotinic acid and quinolinic acid were determined in the filtrates by the differential assay with *Lactobacillus arabinosus*(8) and in some cases by the cyanogen bromide-aniline method. For the 24-hour incubation experiments, one

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or 2 g of rat or hog liver slices was suspended in 3 ml of 0.9% sodium chloride and 5 ml of M/15 phosphate buffer, and incubated in 50 ml Erlenmeyer flasks with shaking. In some cases, one or 2 drops of toluene was added to the flasks at periodic intervals and found to have no measurable effect on the results. All substrates were added in a dry state at the beginning of the incubation.

In Table I are presented the results of some typical experiments. For purposes of comparison, all figures were converted to the same weight of tissue. In no case was there any significant production of nicotinic acid from any of the substrates used. Only 3-hydroxyanthranilic acid was transformed to quinolinic acid under the conditions of these experiments. The larger quantity of tissue slices and longer period of incubation did not appear to influence the results, since the conversion of 3-hydroxyanthranilic acid was essentially complete in as little as 30 minutes. As in previous work(1), there was no evidence for the *in vitro* decarboxylation of quinolinic acid to form nicotinic acid. Results with the chemical method of analysis agreed with those obtained with the microbiological assay, *i.e.*, there was no net production of nicotinic acid with any of the substrates used.

Rat growth and excretion studies. Two experiments were conducted to determine whether or not 3,4-dihydroxyanthranilic acid could replace tryptophan in supporting the growth of rats on a low tryptophan-niacin diet. Female rats from the Biochemistry Division Colony approximately 4 weeks of age were used. In the first experiment, the supplements were incorporated into the diet at a level of 0.25 mM per 100 g of diet. In the second, 0.025 mM per day was injected intraperitoneally. Control animals received the same volume of isotonic glucose solution. The basal ration contained 9% casein, 0.2% L-cystine and 0.2% DL-threonine(9).

TABLE II. Growth Promoting Effect of Tryptophan and 3,4-Dihydroxyanthranilic Acid for Nicotinic Acid Deficient Rats.

		Growth, g/wk	
		Exp. I	Exp. II
Group	Diet	Supplement in ration,* g	Supplement by I.P. inj. daily,† g
1	Basal ration	3	3
2	Basal ration + DL-tryptophan	19	20
3	Basal ration + 3,4-dihydroxyanthranilic acid	6	2

* .25 mM per 100 g of diet.
† .025 mM per day in isotonic glucose solution.

TABLE III. Excretion of Nicotinic Acid and Quinolinic Acid by Rats Receiving Tryptophan and 3,4-Dihydroxyanthranilic Acid.

Group	Daily supplement (I.P. inj.)	N.A., μg/day	Quinolinic acid, μg/day
1	0	3	25
2	.025 μM DL-tryptophan	16.5	71
3	.025 μM 3,4-dihydroxyanthranilic acid	4.8	17

The growth rates obtained in both studies are shown in Table II. It is evident that 3,4-dihydroxyanthranilic acid did not promote growth above that observed for the control groups, while tryptophan supported growth in the normal range for rats on low protein diets. After a 4-week growth period, three of the five rats receiving 3,4-dihydroxyanthranilic acid were fed for one week the same diet supplemented with 100 mg of DL-tryptophan per 100 g of diet while the 3,4-dihydroxyanthranilic acid injections were continued. These animals gained an average of 17 g during this 7-day period, while 2 animals continued on the same regimen without added tryptophan gained 0.5 g during the same period. These results indicate strongly that the slow growth of the rats receiving the compound in question could not have been the result of toxicity overcoming a growth response.

Urine was collected for a 3-day period during the 3rd week of the growth experiment. Microbiological analysis for nicotinic acid and quinolinic acid confirmed the growth data. Whereas the tryptophan injections elevated the quinolinic acid and nicotinic acid excretion 3-4 fold, 3,4-dihydroxyanthranilic acid in equimolar quantities did not alter the urinary excretion values significantly. (Table III).

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Growth tests with Xanthomonas pruni. A strain of the plant pathogen *X. pruni* has been studied and found to respond to all of the postulated nicotinic acid precursors as far back as anthranilic acid(7). It seemed likely that if 3,4-dihydroxyanthranilic acid were an intermediate between 3-hydroxyanthranilic acid and nicotinic acid, it should support growth at levels equal to or less than those at which 3-hydroxyanthranilate is active.

Whereas 3-hydroxyanthranilic acid supported optimum growth at a concentration of 1.2 μM and quinolinic acid at about 200 μM with measurable growth at much lower concentrations, 3,4-dihydroxyanthranilic acid did not show significant activity at levels from 0.12 to 240 μM. Addition of similar concentrations to cultures containing sufficient nicotinic acid (.04 μM) for sub-optimum growth (60-70% transmission) resulted in no growth promoting activity. Some inhibition of growth under these conditions was noted when the 3,4-dihydroxyanthranilic acid concentration was above approximately 100 μM.

To eliminate the possibility that all of the 3,4-dihydroxyanthranilic acid was destroyed during the lag phase of growth, it was added to cultures already actively growing on sub-optimum concentrations of nicotinic acid. Again no growth promoting activity was noted.

Discussion. The explanation of the wide discrepancy between these results and those presented by Makino *et al.*(5) is not known. If 3,4-dihydroxyanthranilic acid were an intermediate as suggested by their data, the intact animal would be expected to utilize it for growth and to excrete nicotinic acid and quinolinic acid as it does when given tryptophan, kynurenine, 3-hydroxyanthranilic acid and quinolinic acid. Likewise, the bacterium tested should respond. The ease with which 3-hydroxyanthranilic acid is converted to quinolinic acid *in vitro* would suggest that any intermediate involved should give rise to large amounts of quinolinic acid when added to such systems. The intermediate reported by Bokman and Schweigert is not 3,4-dihydroxyanthranilic acid‡(10). This intermediate shows an absorption band at approximately 315 mμ in acid solution and 360-375 mμ in buffer at pH 7.4. In trichloracetic acid or ethanol, 3,4-dihydroxyanthranilic acid has two absorption maxima; one at 268 mμ and one at 322 mμ.

Summary. 3,4-dihydroxyanthranilic acid was not converted to quinolinic acid or nicotinic acid by rat or hog liver preparations. Spectrophotometric evidence indicates that this compound is not the transient intermediate formed in the conversion of 3-hydroxyanthranilic to quinolinic acid by liver enzymes. It was not utilized by *Xanthomonas pruni* for growth, or by the rat for growth or formation of urinary nicotinic acid or quinolinic acid.

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‡ In a private communication, Dr. Schweigert states that rat liver preparations failed to form quinolinic acid or nicotinic acid when incubated with 3,4-dihydroxyanthranilic acid supplied by Dr. Makino.

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Mechanics of Cell Division. I. The Living Spindle. (19098)

THEODORE N. TAHMISIAN. (Introduced by A. M. Brues.)

From the Division of Biological and Medical Research, Argonne National Laboratory, Chicago

The destruction of the longitudinal half of a mitotic spindle by X irradiation has been described(1). Chromosomes in the irradiated area of the spindle became pycnotic whereas those in the structurally intact area maintained their separate identities (Fig. 1A).

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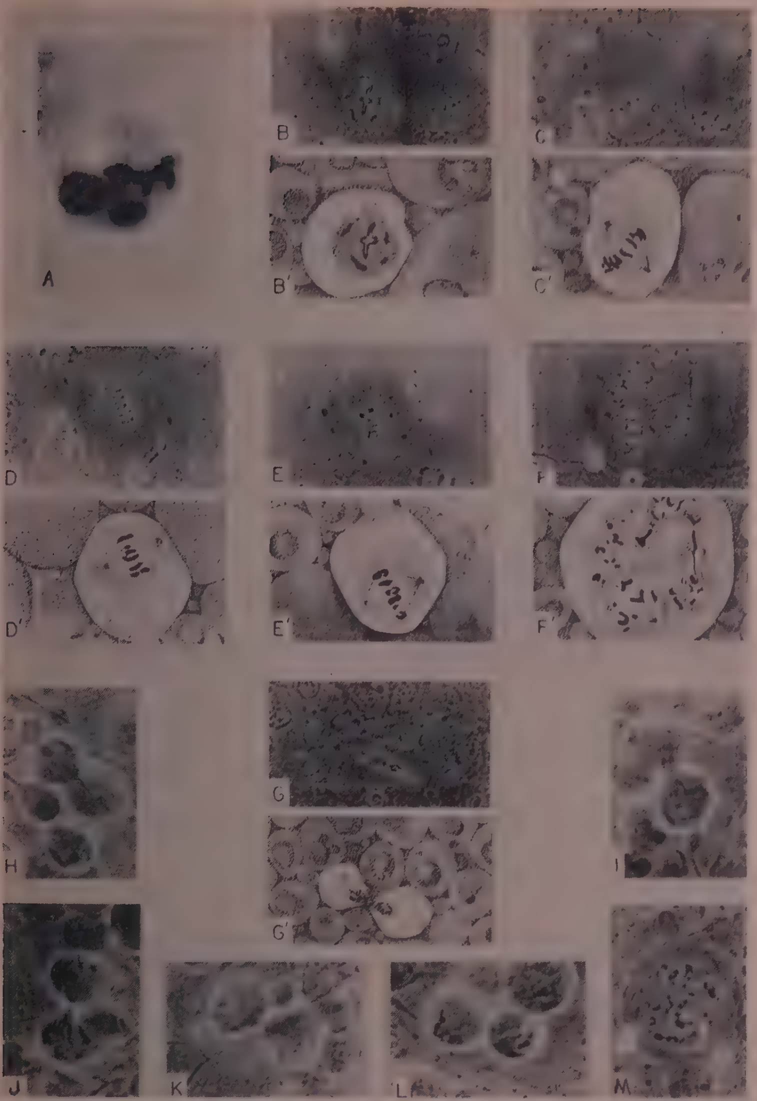

FIG. 1. A. Cell from prediapause embryo that was irradiated with 250 rep emanating from SR^{89}. Note the pycnotic chromosomes in the area where the spindle has been destroyed while the chromosomes that are attached to the remaining portion of the spindle appear intact (× 600). B-G. Phase contrast photomicrographs of living germ cells showing various stages of meiosis and birefringence of the spindle (× 600). B'-G'. Drawings of photographs B, C, D, E, F, and G showing the relationship of the intracellular components with the spindle (× 750). H-M. A phase contrast series of photomicrographs showing a germ cell undergoing meiotic division (× 600). M. A cell that was pretreated with colchicine 24 hr previous to study. Note the absence of a spindle.

Because this work and that of other investigators(2) indicates that a functional spindle is necessary to maintain the orientation of the chromosomes, it was thought important to determine whether the cell and spindle are living when observed under conditions of the

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technics used and to observe the presence of the spindle in the normal cell and its absence in the colchicine-treated one.

Material and methods. Testes from third instar *Melanoplus differentialis* nymphs were used. The nymphs were immersed in 70% ethyl alcohol and then wiped dry with Kleenex. The wings and legs were removed and a dorsal slit was made in the abdomen. The testes were removed under a dissecting microscope with a pair of forceps and placed in Belar(3) solution. The constituents of Belar solution were either autoclaved or passed through a sterile Berkefeld filter, porosity N, prior to mixing. In both cases the calcium and, when used, magnesium solutions were added after almost complete dilution in order to avoid precipitation with phosphate and carbonate. The carbohydrate content was kept at or below 0.2% because cells in their normal environment are bathed in saline of balanced salts in which the carbohydrate content seldom exceeds this value. The pH of the final solution was checked with a glass electrode Beckmann pH meter. The pH of Belar was 6.8. (The pH of grasshopper blood is 6.8 as determined by the Beckmann one-drop glass electrode). After immersion into Belar solution the testicular follicles were placed on a glass slide, and the proximal ends cut and discarded. The distal ends of the follicles, containing cells in the primary and secondary meiotic stages of division, were gently ruptured by mild pressure on the coverslip. Sufficient solution was used to fill the coverslip area without any overflow and the coverslip was then sealed to the slide with paraffin to eliminate evaporation. The observations and the photomicrographs were made with the aid of an Ortholux microscope. This microscope was adapted to the American Optical Co. phase contrast objectives as well as to the phase contrast substage condenser. Spindle birefringence was best observed and photographed approximately 5000 to 6000 A. This range of the visible spectrum was obtained by lowering the substage condenser until a yellow-green ring surrounded the retardation plate of the phase contrast ocular telescope.

Living nymphs were injected with 50 γ of colchicine in 0.1 cc of H_2O in the abdominal cavity 24 hours previous to sacrifice. The approximate concentration of colchicine per nymph was 1.1×10^{-5} g. In no case was a spindle present in the colchicine-treated testes (Fig. 1M). If colchicine was added to the coverslip preparation after the spindle was formed dissolution of the formed spindle did not occur.

Results. The meiotic spindle of the first and second maturation divisions in the grasshopper testis are shown in Fig. 1, B-G and H-L. The spindle fibers are shown to be attached from the centrosome to the chromosome (Fig. 1C, D, F, and F′). This condition is readily observed but difficult to photograph. In order to see the spindle it was necessary to find portions of the slide in which the cells were only one layer thick.

The cells that appear in the photographs (Fig. 1, B-G) were under pressure. Meiosis in these cells progressed slightly beyond metaphase. Fig. 1, H-L shows that cells that were under less pressure divided; the interzonal fibers and the *Zwischenkörper*(4) can be seen in these pictures. The mitochondria can also be seen as long strands occupying positions lateral to the spindle axis. In late anaphase or early telophase they were seen to move across the spindle to a position located centrally between the two newly-forming nuclei and become aligned along the axes of the spindle fibers. Such conditions are shown in Fig. 1G and H-L. Colchicine-treated material observed under identical conditions did not show spindles (Fig. 1M).

Discussion. Although most cytologists agree that the spindle is a real entity in the living cell(5-11), there are those who believe it is

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of an artificial nature. Several recent reviews on the subject are available(12,13). Under conditions approximating as nearly as possible the *in vivo* situation, the spindle was photographed in an uninjured condition in living material, and the cells were seen to complete division. It is assumed that spindle formation is not an artifact produced by pressure, since colchicine-pretreated cells observed under similar conditions did not have spindles.

Rosza and Wyckoff(14) showed that in acid- or Flemming-fixed material the interzonal fibers were apparent in onion root tip, whereas they were absent in neutral formalin-fixed material. On the basis of our observations their findings on acid-fixed material may be considered "realfacts"(15) and the dissolution of the fibers by neutral formalin may be considered as an artifact. For the same reason the electron micrograph of the spindle prepared by Beams *et al.*(16) may be considered to be a "realfact" and not an artifact. Since we have observed and photographed the centriole, spindle fibers, and interzonal fibers, in living material, we conclude that a spindle in fixed tissue is a "realfact."

Summary. The observation that exposure to X irradiation causes partial dissolution of cell spindles and that chromosomes in the destroyed area become pycnotic led to this study of the living spindle. Photographs of grasshopper testis, living cells that have been immersed in Belar solution show spindle fibers and the attachment of some of these fibers from the centrosome to the chromosome. The reality of the spindle in fixed tissue, as revealed by phase contrast microscopy, is emphasized as a result of these studies. Prior to this experiment, Schmitt and Belar were also able to show the birefringence of spindle fibers.

The author is indebted to Drs. H. W. Beams and A. M. Brues for reviewing this paper.

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Detection by Tissue Culture of an Organism Resembling *Histoplasma capsulatum* in an Apparently Healthy Horse.[*] (19099)

CHARLES C. RANDALL, MARY F. ORR, AND FRED G. SCHELL.
(Introduced by Roy C. Avery.)

From the Department of Pathology, Vanderbilt University School of Medicine, Nashville, Tenn.

In this laboratory we have been concerned with the cultivation of the virus of equine abortion (E.V.A.) in certain tissues of the horse maintained *in vitro*(1). During the course of the investigation, intracellular organisms, resembling *Histoplasma capsulatum*, were observed in supposedly healthy horse tissue grown in tissue culture. As a result of these findings, Randall and McVickar(2) demonstrated the experimental development of *H. capsulatum* in tissue culture.

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[*] This investigation was supported in part by a grant from the Grayson Foundation.

1. To be published.

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The present paper deals with the detection by tissue culture methods of an intracellular parasite apparently indistinguishable from *H. capsulatum*. The observation is of interest, because the method is unique from the standpoint of diagnosis and histoplasmosis is a rarity in the horse, only one (unillustrated) case report(3) being found in the literature. It should be noted that the present paper is an outgrowth of the above problem(1), and only the pertinent details will be given here.

Experimental procedures. Horse tissues were obtained in the following manner: an apparently healthy 5-year-old mare of about 6 months gestation was instantaneously killed by a shot through the head; adult and fetal tissues were obtained with scrupulous attention to aseptic technic and sterile instruments were changed for each tissue. Portions of fetal lung, liver, spleen, heart; adult lung, liver, and spleen; that portion of the placenta with central mesoderm bordered by allantois on one side and chorion on the other and the amnio-allantoic membrane were taken for tissue culture. Representative portions were placed in Zenker-acetic acid for control studies. For reference, samples of each tissue were stored in a deep freeze at –40°C. All organs, fetal and adult, appeared normal grossly. All tissues were cultivated on cover-glasses placed in test tubes, and in addition, fetal spleen, lung, and liver were cultivated by the flask method(4). Chick embryo extract (E.E.) and chicken plasma were prepared by conventional methods. The supernatant or liquid phase consisted of 50% human ascitic (cancer) fluid, 47% Hanks' balanced salt solution (B.S.S.) and 3% E.E., containing 500 units of penicillin per ml. The pH of the mixture was adjusted to 7.6. Cover-glass cultures were fixed in Zenker-acetic acid and either stained *in situ* by the hematoxylin-eosin-azure method (H.E.A.), or were cut out of the clot and routine H. and E. stained paraffin sections were prepared. The ingredients of the clot and supernatant were cultured on blood agar and Brewer's thioglycollate medium for 10 days at 37°C without growth. As a number of the cultures were inoculated with E.V.A., the preparation of this material is mentioned, so as to eliminate a possible source of contamination. Virus containing material was centrifuged for 1 and ½ hours at 13,500 rpm at 4°C and 0.5 to 2 ml aliquots were cultured on the above media for 2 weeks. The technic was such as to preclude the likelihood of *H. capsulatum* being a contaminant.

The following methods were used in preparing cultures of the various adult and fetal tissues. The tissues were cut in 2 mm squares and immediately rinsed thoroughly with B.S.S. (A) *Flask method.* Three parallel sets of flasks were prepared with fetal lung, spleen, and liver, respectively. Four to 8 pieces of tissue in a minimal amount of fluid, were transferred in a 5 ml pipette to a 50 ml Erlenmeyer flask containing 3 ml of supernatant, incubated at 37°C, and observed daily. A certain number of cultures were inoculated with 0.03 ml of the virus suspension within the first 24 hours of incubation. Tissues were fixed for sectioning after 5 and 8 days of incubation. (B) *Cover-glass method.* Three or 4 pieces of tissue were embedded in a plasma clot (equal parts of chicken plasma and E.E.) on a No. one 12 x 50 mm cover-glass. The preparation was then introduced into a 16 x 150 mm pyrex test tube, and after standing for one hour, 2 ml of supernatant was added to each tube, and the cotton plug was replaced by a sterile rubber stopper. The cultures were slanted in a test tube basket at an angle which allowed the supernatant barely to cover the surface of the cover-glass, and incubated at 37°C. The cultures were observed daily for bacterial contamination and pH change. The clear supernatant was changed once weekly. At the end of the 14 days growth period, about ½ of each of the various sets of tissues was inoculated with the virus suspension, with the remainder considered as controls. Cultures were fixed and stained by H.E.A. method consecutively for 5 days, when the experiment was terminated. On the last day several controls and inoculated cultures of each tissue were also cut

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3. Richman, H., *The North Amer. Vet.*, 1948, v29, 710.

4. Parker, R. C., *Methods of Tissue Culture*, Paul B. Hoeber, Inc., 1950.

out of the plasma clot, fixed in Zenker-acetic acid and H. and E. paraffin sections were prepared.

Results. (A) *Flask method.* Thorough examination of the numerous H. and E. stained sections failed to demonstrate any evidence of infection.

(B) *Cover-glass method.* The numerous explants of adult horse spleen stained by the H.E.A. method showed fair outgrowth of fibroblast-like cells. In 2 such 19-day-old spleen cultures, one inoculated with E.V.A., and one a control, a very rare cell contained intracellular organisms identical morphologically with the yeast cells of *Histoplasma capsulatum.* Numerous preparations of the other adult and fetal tissues appeared free of such forms. The results obtained from another group of cultures grown on film coated coverslips and subsequently cut from the clot, were most interesting. A study of two 19-day-old control cultures, one of adult spleen and one of amnio-allantoic membrane, revealed that the majority of cells contained many intracellular forms, identical with those described above (Fig. 1). Irrespective of the preparation, none of the infected cells showed any stigmata of degeneration.

When the intracellular forms were first noted in stained preparations the tissues that had been stored for 6 weeks in the deep freeze (with the exception of placental tissue) were ground with mortar and pestle and cultured on plasma agar at 37°C and room temperature. No growth occurred. Unfortunately all the tissue culture preparations had been fixed and stained. Attempts to demonstrate by histological study that the organism was present in the tissues removed at autopsy, also met with failure.

Discussion. A study of adult horse spleen and amnio-allantoic membrane cultivated by the cover-glass method, has revealed that many cells of the explant contained yeast cells in the cytoplasm apparently morphologically identical with *H. capsulatum.* Control H. and E. stained paraffin sections of autopsy material appeared free of infection. This fact, coupled with the demonstration that numerous cells of tissue culture material contained intracellular forms, seems to indicate that multiplication of such magnitude took place so as to make their detection possible by microscopic study. The organism was found only intracellularly, and the infected cells appeared healthy.

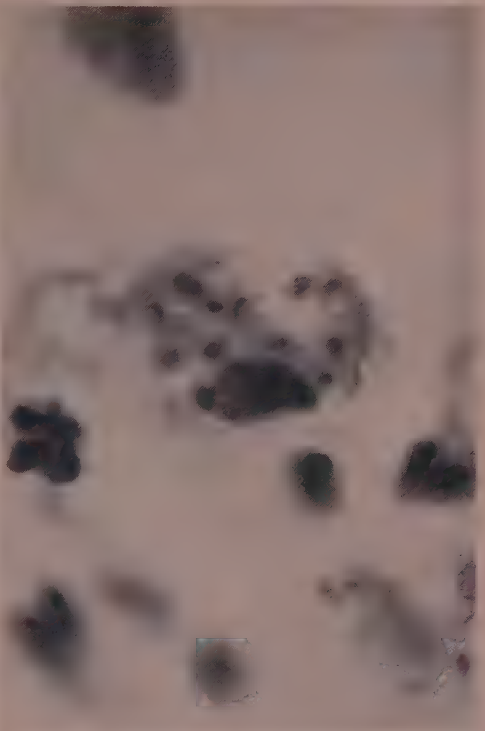

FIG. 1. Nineteen-day-old culture of horse amnio-allantoic membrane. Note the large cell filled with intracellular, apparently encapsulated forms, resembling intracellular yeast cells of *Histoplasma capsulatum.* Many of the cells of the explant are out of focus due to the thickness of the preparation. × 1425.

To our knowledge tissue culture methods have not, heretofore, been used as a method of detecting fungus infections. Histoplasmosis itself is apparently rare in the horse, and the demonstration of intracellular forms in the cells of the amnio-allantoic membrane suggests that placental transfer of *H. capsulatum* is possible.

Mention should be made of a disease of horses and mules known variously as farciminosus, epizootic lymphangitis or pseudofarcy, the causative agent of which is *Cryptococcus farciminosus.* A few doubtful cases have been reported in the United States, but most of these, if not all, were cases of sporotrichosis(5). The clinical signs are characteristic and lack of them would tend to eliminate this possibility in the case in question. Da Rocha Lima(6) first made the interesting observation that the parasitic yeast cells of *Cryptococcus farciminosus* and *H. capsulatum* are very similar. Comparison of the two fungi in tissue culture might establish significant differences.

Summary. Intracellular organisms resembling *Histoplasma capsulatum* have been observed in apparently normal amnio-allantoic membrane and adult horse spleen maintained in tissue culture.

The significance of this finding and comparison with another fungus *Cryptococcus farciminosus* is discussed.

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Improvement of Protein Quality of Whole Wheat.* (19100)

Rachel Beaudoin, Jean Mayer, and Fredrick J. Stare.

From the Department of Nutrition, Harvard School of Public Health, Boston, Mass.

In the course of experiments designed to study the suitability of shredded wheat materials in low sodium diets some interesting observations on the improvement of the biological value of the protein of whole wheat have been made.

Experimental. The biological value of whole wheat products was assayed by the growth response of weanling albino rats fed a standard diet of the following composition: Wheat products 86%, corn oil 8%, cod liver oil 2%, salt mixture(1) 4%, choline 0.1% and contained per kilo of diet, thiamine 4 mg, riboflavin 8 mg, nicotinic acid 40 mg, calcium pantothenate 40 mg, pyridoxine 4 mg, biotin 1 mg, folic acid 0.5 mg, α-tocopherol 40 mg, and menadione (vit. K) 4 mg. On this diet the only variable element was the protein component (wheat product). Five groups of eight weanling albino rats, comparable as to weight, were put on the following diets: Control group: whole wheat; assay groups: whole wheat cooked; whole wheat cooked and fan dried; whole wheat cooked, fan dried, shredded, baked and oven-dried—commonly known as "shredded wheat"; comparison group: white flour. All of these wheat preparations were fed at a level of nitrogen such that they furnished 8.6% protein. Slight variations in the per cent of wheat product in the diet as a result of varying nitrogen content were equalized by the addition of the proper percentage of glucose. The rats were housed in individual cages and fed the above diets and water *ad libitum* for a period of five weeks. Records were kept of the daily food intake and the animals were weighed twice a week. The essential details of the heat treatment of the whole wheat are as follows: cooked, the whole wheat is cooked in boiling water for 38 minutes; fan dried, the cooked product is dried in a rapidly moving current of air at a tempera-

TABLE I. Average Gain of Wt of Weanling Rats Fed Wheat Products at Various Stages of Processing.

Basal diet with the following sources of protein, all fed at a level of nitrogen to give 8.6% protein	Avg gain in wt, g
Whole wheat	15.4
" " cooked	40.8
" " " and fan dried	24.8
" " " " " ", shredded, baked and oven dried = shredded wheat	30.5
White flour	10.4

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* This investigation was supported in part by a grant-in-aid from the Nutrition Foundation, New York, and the National Heart Institute, National Institutes of Health, Public Health Service.

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TABLE II. Statistical Analysis of the Biological Value of Wheat Protein.

Comparison of the diets	Degrees of freedom	Observed ''t''	Probability*
Whole wheat vs. whole wheat cooked	11	3.52	<.01
’’ ’’ cooked vs. shredded wheat	12	1.38	.2
’’ ’’ ’’ and fan dried vs. shredded wheat	14	1.16	.3 –.2
’’ ’’ vs. white flour	13	2.96	.02–.01

* When the probability of getting a ''t'' value at least as large as the observed ''t'' is .05 or less, the difference in wt gain is considered significant.

ture of 28°C for 12 hours; shredded, the fan dried material is shredded, baked in an oven at 250°C for 18 minutes and then dried to a moisture content of 5% by holding at 150°C for approximately 65 minutes.

Results. The average gain of weight for each of the groups at the end of this experiment is given in Table I. These results were compared and analyzed and the "t" test was used as a measure of significance(2).

It can be seen from Tables I and II that: (a) as is well known, whole wheat has a biological value which is significantly superior to that of white flour; (b) there is a further significant improvement in the biological value of the protein of the whole wheat when this whole wheat is subjected to cooking in boiling water; (c) there is no significant alteration of the biological value of the protein of cooked whole wheat when it is made into shredded wheat biscuits.

Discussion. Heat processing is known to affect the nutritive value of proteins in various ways. The adverse effects of heat on dietary proteins have been reviewed in a recent publication(3). While it is well known that in addition to soy beans(4) many other leguminous proteins improve in nutritive value after the application of moist heat, the same effect has not been reported in the case of cereals. It is a generally accepted view, however, that when cereals are properly cooked no undesirable changes in the protein component occur and their nutritive value appears to be equal to that of the unheated protein(5).

This study indicates that the cooking of whole wheat grain in the commercial preparation of shredded wheat results in a significant improvement of its original growth promoting value. This marked increase in nutritive value is not altered to any significant degree in the final processing of the product. So far as we are aware, this effect of moist heat on whole wheat has never been reported. The reason for this increase in nutritive value is not known.

In the case of legumes where similar phenomena have been observed various hypotheses have been advanced: removal of a trypsin inhibitor, elimination of toxic substance, or direct action on the protein making certain amino acids more available.

Shredded wheat has a sodium content of 2 mg per 100 g, which is the same as that of polished and coated rice(6). Because of its low sodium content, the high quality of the protein of shredded wheat, its richness in minerals and vitamins of the B complex, it should be a useful food in introducing variety in low sodium diets.

Summary. Experiments are reported showing that the cooking of whole wheat as in the preparation of shredded wheat results in a significant improvement in the nutritional value of the whole wheat. This improvement is not lost when the material is fan-dried or oven-dried in the preparation of shredded wheat.

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Influence of Sex Hormones on Citrate Synthesis in Liver.* (19101)

KENNETH P. DUBOIS, KENNETH W. COCHRAN, AND MARCELLA M. ZERWIC.
From the USAF Radiation Laboratory† and the Department of Pharmacology, University of Chicago.

Recent studies in this laboratory(1) have indicated that citrate synthesis in the liver is normally under some regulatory influence by the sex hormones. Through the use of the fluoroacetate technic developed by Potter(2) for studying citrate synthesis *in vivo* we found(1) that the livers of normal female rats accumulate large quantities of citric acid in contrast to the inability of normal male rats to accumulate citrate in the liver(3,4) following fluoroacetate treatment. The present investigation was undertaken to obtain information on the factors responsible for the sex difference in citrate synthesis in the livers of fluoroacetate-treated rats. The present communication describes the results of measurements of citrate synthesis in the livers of castrated, adrenalectomized, and immature male and female rats as well as the effects of administration of testosterone and estradiol on citrate formation in the liver. These measurements indicate that androgens suppress citrate synthesis in the liver and that procedures which decrease androgen synthesis will release intermediary carbohydrate metabolism in the liver of male animals from this hormonal control.

Materials and methods. Male and female Sprague-Dawley rats weighing 175 to 250 g were used unless otherwise specified. The fluoroacetate technic developed by Potter(2), in which rats are given 3.5 mg/kg of purified sodium fluoroacetate intraperitoneally and sacrificed 3 hours later, was employed for measuring citrate synthesis *in vivo*. Citric acid was determined by the procedure of Natelson *et al.*(5) using the modifications recommended by Potter and Busch(3). Extra animals were included in each group to allow for possible early deaths from the fluoroacetate treatment. This was especially necessary after treatment with high levels of testosterone which seemed to increase susceptibility to fluoroacetate. Commercial solutions of testosterone propionate and alpha estradiol dipropionate in sesame oil were administered subcutaneously.

TABLE I. Citrate Accumulation in Livers of Castrated Rats After Fluoroacetate Treatment.

Days after castration	μg citric acid/g fresh tissue	
	Males	Females
Control	64 (44–71)	616 (248–1540)
8	369 (178–739)	895 (494–1285)
14	221 (144–384)	435 (288–520)
28	315 (224–488)	305 (176–472)

Results. In previous experiments we found (1) that the livers of normal female rats contained 248 to 1540 μg of citric acid/g of tissue in 3 hours after fluoroacetate administration whereas after similar treatment the livers of male rats contained less than 100 μg of citric acid/g(3,4). To ascertain whether this sex difference is explainable on the basis of stimulation of citrate synthesis by estrogens or inhibition of the synthesis by androgens experiments were performed on castrated male and female rats. The results of citrate measurements on the livers of fluoroacetate-treated rats sacrificed at various intervals after removal of the testes and ovaries are shown in Table I in which the average value and the range for each group of 4 to 6 animals are presented. These measurements demonstrated that castration of female rats does not alter

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† Formerly the University of Chicago Toxicity Laboratory.

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the ability of the liver to accumulate citrate following fluoroacetate treatment whereas male rats acquired the ability to accumulate citrate in the liver following castration. It thus appears that castration of male rats releases intermediary carbohydrate metabolism in the liver from a regulatory action by androgens. No sex difference in citrate accumulation was observed in spleen and thymus of normal rats and castration did not alter the values for these tissues from the normal range.

To ascertain whether adrenal steroid hormones influence citrate synthesis in the liver male and female rats were adrenalectomized, maintained on 0.9% sodium chloride for 2 days, and were then treated with fluoroacetate (2). The livers of 4 adrenalectomized male rats accumulated 404 (232-704) μg citrate/g of tissue in contrast to values below 100 μg/g for normal male rats. The livers of adrenalectomized female rats accumulated citrate after fluoroacetate treatment and thus did not differ from normal female rats in this respect.

Following indications that androgens inhibit citrate accumulation in the livers of fluoroacetate-treated rats, testosterone propionate (2 mg/kg/day) was administered subcutaneously to normal female rats and groups containing 6 animals were then given fluoroacetate at intervals from 2 to 12 days after beginning the hormone treatment. No significant decrease in citrate accumulation was observed in the liver, spleen, and thymus during the first 3 days of treatment with testosterone. However, in 6 days the average citrate accumulation in the liver decreased from the normal of 616 μg/g to 297 μg/g. After 12 days of treatment with testosterone the livers of female rats accumulated an average of 166 μg of citrate/g and none of the animals within the group accumulated more than 174 μg of citrate/g, thus indicating inhibition by testosterone of citrate synthesis in the livers of female rats. After 12 days of treatment with testosterone, citrate synthesis in spleen and thymus was inhibited 37% and 24% respectively, and marked atrophy of the thymus occurred. In another experiment groups of female rats were given 0.1, 0.5, 1.0 and 2 mg/kg of testosterone propionate for 12 days after which time fluoroacetate was administered and citrate analyses were performed on liver, spleen, and thymus from at least 4 animals treated with each dose of testosterone. The average citrate accumulation in the liver following administration of fluoroacetate was 206, 120, and 197 μg/g of tissue for the groups receiving 0.5, 1, and 2 mg/kg of testosterone propionate daily. None of the values for individual animals in these groups exceeded 300 μg/g of tissue. Daily administration of 0.1 mg/kg of testosterone propionate for 12 days did not significantly decrease citrate synthesis in the liver and at 14 days after a single dose of 10 mg/kg of testosterone no alteration in the ability of the livers of fluoroacetate-treated female rats to accumulate citrate *in vivo* was noted. These experiments demonstrated that after treatment of female rats with testosterone for several days citrate accumulation in the liver was inhibited and approached the values for normal male rats.

Although the experiments described above indicated that estrogens do not exert a direct stimulatory effect on citrate synthesis in liver, the administration of estradiol to normal male rats appeared to antagonize the inhibitory effects of androgens. Thus, when male rats were treated with 1 mg/kg of estradiol dipropionate for 3 days and were then given fluoroacetate 7 days later, citrate accumulation occurred in the liver as evidenced by an average value of 259 (228-316) μg of citrate/g of tissue for 4 animals. In another experiment two groups each containing 6 male rats were given single doses of 0.5 and 1 mg/kg of estradiol dipropionate and sacrificed 14 days later. The livers of these animals accumulated 348 (248-600) and 388 (56-744) μg citric acid/g of tissue respectively after fluoroacetate treatment. Spleen and thymus values were unaffected by the estradiol treatment. These results indicate an antagonistic action between estrogens and androgens on citrate synthesis in the liver *in vivo*. This antagonism, however, might result from suppression of pituitary activity and consequent inhibition of androgen synthesis by the testes rather than a direct antagonism in the liver.

In previous studies(1) we found that 50 g

TABLE II. Citrate Accumulation in Tissues of Immature Female Rats After Fluoroacetate Treatment.

Age in days	Citric acid (μg/g fresh tissue) Liver	Spleen	Thymus
24	56 (46–62)	992	644
29	178 (64–336)	884	840
35	202 (88–304)	1019	996
42	398 (184–616)	1125	950
49	768 (656–880)	1172	976

female rats (24 days old) were unable to accumulate citrate in the liver following fluoroacetate treatment. To obtain information regarding the time of onset of citrate accumulation in the livers of female rats groups each containing 4 animals were treated with fluoroacetate and sacrificed at intervals from the ages of 24 to 49 days. The results of citrate measurements on tissues from these animals are shown in Table II. It was necessary to pool spleen and thymus glands for each group to obtain enough tissue for analysis. Citrate accumulation began to occur in the livers of young female rats at the age of 29 days which is prior to the time of onset of estrogen production(6) giving further indication that estrogens are not directly involved as stimulants of citrate synthesis in the liver. The low values for liver at the age of 24 days may be related to the lower concentration of respiratory enzymes in neonatal life which was demonstrated by Potter *et al.*(7) since citrate synthesis in spleen and thymus was also below the normal rate for adult animals. A similar experiment was carried out on young male rats and at 29 and 35 days of age the citrate values for liver were slightly elevated above the normal range for adult animals as evidenced by average values of 158 and 133 μg/g of tissue. After the age of 35 days the values closely approximated those obtained for adult male rats.

The inhibitory action of physiological concentrations of androgens on citrate synthesis in the liver *in vivo* is apparently lost when the tissue is homogenized because the livers of normal male rats are capable of synthesizing large quantities of citrate *in vitro*(3). We have observed, however, that testosterone[‡] (150 μg/Warburg flask in 0.02 ml of ethanol) inhibits oxygen consumption and citrate formation *in vitro* to the extent of about 30% and 50% respectively, when pyruvate plus fumarate are used as substrates with 50 mg of homogenized liver according to the procedure of Pardee and Potter(8). This inhibitory effect by testosterone is in agreement with the evidence obtained from *in vivo* experiments that testosterone suppresses citrate accumulation in the livers of fluoroacetate-treated rats and more extensive studies on the nature of this inhibitory action are, therefore, being conducted.

Discussion. The present investigation has provided information supplementary to our previous experiments(1) which demonstrated a sex difference in the response of the liver to fluoroacetate *in vivo*. The absence of a sex difference in citrate synthesis in other tissues such as spleen and thymus suggests that the factors responsible for the difference in citrate synthesis in the livers of male and female animals do not normally affect citrate synthesis in other tissues. Retention of the ability of fluoroacetate-treated female rats to accumulate citric acid in the liver following castration seems to eliminate the possibility that estrogens stimulate citrate synthesis and the ability of young female rats to accumulate citrate in the liver prior to the onset of estrogen production gives further indication of the absence of a stimulatory action by estrogens. Implication of androgens as inhibitors of citrate synthesis was indicated by the occurrence of citrate accumulation in the livers of castrated male rats following fluoroacetate treatment and by the depressant action of injected testosterone on citrate synthesis in the livers of female rats. With respect to the latter point the necessity for continued administration of testosterone to female rats

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for several days requires further study. Acquisition of the ability of male rats to accumulate citrate in the liver after adrenalectomy suggests that adrenal cortical hormones also exert a regulatory influence on citrate synthesis in liver and the similar response obtained after feeding male rats desiccated thyroid(2) suggests that several hormones may be involved in the regulation of citrate synthesis in the liver.

The exact manner in which androgens influence the response of the liver to fluoroacetate requires further investigation. However, the present studies have provided a basis for subsequent experimentation on the influence of androgens on intermediary carbohydrate metabolism. The available data indicate that testosterone inhibits the formation of citrate from pyruvate or acetate in the liver and this effect may be of significance in connection with the basic mechanism underlying the metabolic action of the sex hormones. The alteration in the metabolic pattern of the liver which we previously observed(1) in X-irradiated male rats may be due to a release of the liver from the regulatory action of androgens through suppression of steroid synthesis.

Summary. A study of factors influencing citrate synthesis was conducted using the fluoroacetate technique of Potter(2) to determine the cause of the marked sex difference(1) in citrate accumulation in the liver following administration of fluoroacetate to rats. Castration of female rats did not decrease citrate synthesis in the liver but castrated and adrenalectomized male rats acquired the ability to accumulate citric acid in the liver after fluoroacetate treatment. Administration of testosterone to female rats for several days decreased citrate synthesis in the liver and testosterone inhibited citrate synthesis by rat liver homogenates *in vitro*. The results of these experiments indicated that androgens suppress citrate formation in the liver.

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Effect of Calcium Status, Mass of Calcium Administered and Age on Ca^{45} Metabolism in the Rat. (19102)

Sam L. Hansard, C. L. Comar, and M. P. Plumlee.

From the University of Tennessee, Atomic Energy Commission Agricultural Research Program, Oak Ridge.

The value of radiocalcium in nutritional studies has been obscured by the lack of agreement between investigators and difficulties of interpretation due to the exchange of Ca^{45} between extracellular fluid and bone (1-5). Observations during the course of nutrition studies with Ca^{45} indicated a surprisingly rapid response of the rat to changes in the calcium content of the diet. It seemed important, therefore, to establish the extent to which conventional experimental procedures were producing a bias in the results obtained by this sensitive method of studying calcium metabolism.

This study shows the effects of the following factors upon the behavior of labeled calcium administered orally to the rat: (a) the actual calcium status of the animal at the time of dosage, (b) the calcium content of the intestinal contents at the time of dosage, and (c) age of the animal. Physiological implications of calcium depletion and the interpretation of Ca^{45} studies are discussed.

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TABLE I. Composition of Experimental Rat Rations (g per 1000 g of Ration).

Ingredients	Diet Ca-1 (Low)	Diet Ca-3 (Normal)
Corn starch	655	643
Casein (vitamin-free)	180	180
Irr. yeast*	10	10
UT-AEC min mix†	20	20
Cod liver oil‡	2	2
Wesson oil	40	40
Cellulose	40	40
Vitab§	40	40
KH_2PO_4	13.2	13.2
$CaCO_3$	0	12.2
% calcium (by analysis)	.013	.5
% phosphorus ”	.4	.4

* Irr. yeast contained 360 I.U. vitamin D/g.

† UT-AEC basic mineral mixture No. 1 is a modification of the calcium-phosphorus free mixture used by H. G. Day *et al.* (*J. Nutr.*, 1938, v16, 525) and includes the following minerals: NaCl 39.84, KCl 18.2, $KHCO_3$ 26.2, $MgSO_4$ (anhyd) 12, $CuSO_4 \cdot 5H_2O$ .4, $MnSO_4 \cdot 4H_2O$ .2, $ZnSO_4 \cdot 7H_2O$ .6, $FeCl_3 \cdot 6H_2O$ 2.5, $K_2SO_4 \cdot 2H_2O$ .02, KI .03 and NaF .01 %.

‡ Cod liver oil contained 90 I.U. vit D and 900 I.U. vit A/g.

§ Vitab, available from Nopco Chemical Co., Harrison, N. J.; fortified with .4 mg riboflavin per g immediately preceding mixing.

Methods. These experiments were carried out with highly inbred Wistar rats of both sexes. The young were weaned at 28 days from mothers maintained on commercial rat pellets and, as determined by the experiment, were placed on the normal (Ca-3) and/or low calcium (Ca-1) experimental diets. These diets were identical except for the variation in calcium content (Table I). All rats were fed and watered *ad libitum.* Following the definite pre-experimental periods of preparation on known rations, feed was removed from all cages 4 hours before dosing to insure uniformity of conditions; the animals received a single oral dose of labeled calcium by stomach tube and were placed immediately into metabolism cages equipped for the separation of the urine from the feces for complete balance studies. Unless otherwise indicated, the dosage consisted of 0.5 ml calcium 45 chloride solution containing about 10 microcuries in less than 1 mg calcium with the pH adjusted to 6.0. At sacrifice after 96 hours, fresh tissue samples, feces and urine were taken for routine total calcium and phosphorus determinations and for the measurement of calcium 45 by methods previously reported(6). All values involving labeled calcium have been calculated on an equivalent basis so as to be comparable between animals and experiments.

Effects of changing calcium level of ration. The first study of this series was designed to show the effects of changing the calcium level of the ration at varying times before administration of the labeled calcium. Following the definite pre-experimental period, animals were selected from the low and normal calcium groups and placed on the current intake ration for 48, 96 and 408 hours previous to dosage with Ca^{45} as indicated in Table II. The results show the actual calcium intake and excretion per rat per day during the 96-hour experimental period following oral administration, the percent of labeled calcium excreted, the percent of excreted calcium which was derived from the body stores, and the calcium content and calcium 45 accumulation in the femur shaft. The percent of excreted calcium which was derived from the body stores was calculated without taking into consideration the labeled calcium which reached the body stores and was subsequently excreted during the experimental period. It is considered that this method of expression does not change the overall picture. The important basic effects can be noted from consideration of Lots 1 to 4. As might be expected, Lot 1 rats were in a positive calcium balance, excreting about 50% of the total calcium intake, whereas Lot 4 rats on the low calcium diet throughout, were in negative balance. Lot 2 rats which had been on the low calcium diet for only 48 hours had gone from a positive to a negative balance, whereas Lot 3 rats which had been on a normal diet for only 48 hours, were already in positive balance. In the case of Lot 1, about 30% of the labeled calcium was excreted, whereas in the other three lots, significantly lower amounts were excreted. In line with this it is noted that in Lot 1 rats about 40% of the excreted calcium was derived from the body stores, while with the other groups this value

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TABLE II. Effects of Dietary Calcium Changes Previous to Oral Ca^{45} Administration on Its Excretion and Bone Accumulation (4 rats in each group; 72 days of age).

Lot No.	Hr on current intake	Previous Ca intake	Current Ca intake	mg Ca per day		% excreted			Femur shaft			Ca^{45}
				Intake	Excreted	Total Ca	Ca^{45}	Body* stores	mg Ca/g	Ca^{45}†	Specific‡ activity	Frac. Ca^{45} absorbed
1	48	Normal	Normal	44	22	50	30	40	196	.60	3	.86
2		,,	Low	1.8	1.9	106	6.5	94	170	1	5.8	1.1
3		Low	Normal	45	3.1	6.9	.20	97	85	1.5	17.7	1.5
4		,,	Low	1	1.5	150	.60	99	60	1.5	26.7	1.5
5	96	Normal	,,	1.8	2.1	117	8.3	93	168	.80	4.7	.87
6		Low	Normal	46	.9	2	.24	88	116	1.7	14.2	1.7
7	408	Normal	Low	2.6	.2	7.7	.44	95	140	1.4	10	1.4
8		Low	Normal	49	5.2	10.6	4.8	55	143	1.5	11	1.6

* % of excreted Ca derived from body stores equals $100\left[1-\left(\frac{\%\ Ca^{45}\ \text{excreted}}{100}\right)\left(\frac{\text{mg Ca intake}}{\text{mg Ca excreted}}\right)\right]$.

† μg of labeled Ca per g fresh tissue based on dosage of 10 μg per 100 g body wt.

‡ μg of labeled Ca x 10^3 per g of total Ca based on above dosage.

was from 95 to 100%. In considering the values for the femur shaft it is noted that 48 hours on a low diet had caused an apparently slight decrease in the calcium content (Lot 1 vs. Lot 2), whereas 48 hours on a normal diet had caused an increase (Lot 3 vs. Lot 4). The accumulation of Ca^{45} in the bone was lowest in Lot 1 and significantly higher in the other lots. Greater differences in turnover or specific activity between lots was due to the fact that both the Ca^{45} accumulation and calcium content of the bone entered into the calculation. Lots 5-8 show the same trends accentuated by the longer period on the changed ration. It is of interest in comparing Lots 7 and 8 to note that the calcium balance and bone values were about the same. Nevertheless, the Lot 8 rats, on a current normal intake, were beginning to show an increased excretion rate of Ca^{45} even though the bone calcium content had not yet reached a normal level. The implications of these results are clear: a biased picture of calcium behavior may well be obtained if the dietary calcium is changed radically before administration of the labeled element, or during the balance period.

The physiological aspects are of considerable interest. As will be discussed in more detail later, it can be noted that the mass of calcium in the intestinal contents at the time of absorption of Calcium 45 had little effect on the results obtained. In the case of simple mass effects, one would normally expect a lower excretion value when the mass was lower. Thus Lot 2 rats certainly had a lower intestinal content of calcium than did those of Lot 3, but Lot 2 nevertheless showed a higher excretion: this comparison also holds for Lots 5 and 6. It seems apparent that rats changed from a normal to a low calcium diet rapidly develop an increased capacity for absorption of this element from the tract. In the case of animals maintained on low calcium for longer periods of time (>96 hr) it seemed likely that the calcium status of the bone was reflected in the increased capacity for absorption of this element from the tract. It may be noted from the last column of Table II that the bones of animals on low calcium for longer than 96 hours showed a higher Ca^{45}

TABLE III. Effects of Calcium Depletion on Excretion and Bone Accumulation of Orally Administered Ca^{45} (8 rats in each group; 32 days of age at start of experiment).

Days on low Ca (Ca-1)	% Ca^{45} dose excreted	Femur shaft				Ca^{45}
		Ca^{45}*	mg Ca/g	Specific† activity	% ash	Fraction Ca^{45} absorbed
0	8.8	.71	105	8.7	32	.78
12	.7	1.4	102	13	27	1.4
24	.5	1.6	63.5	25	18	1.6
36	.8	1.7	65	26	17	1.7
48	1.2	1.6	68.5	24	18	1.6
60	.9	1.4	68	21	18	1.6

* μg of labeled Ca per g fresh tissue based on dosage of 10 μg per 100 g body wt.

† μg of labeled Ca × 10^3 per g of total Ca based on above dosage.

uptake in the femur shaft per unit of dose absorbed. This would indicate that in animals on a depletion regime, increases in the ability to absorb calcium from the tract may precede changes in the bone.

In part, the results thus far discussed have been concerned with short-term depletion effects. The following study is concerned with the course of depletion continued over a longer time period. Six lots of 8 weanling rats each were placed on a low calcium ration (Ca-1) and at intervals designated in Table III they were given an oral dose of Ca^{45} and sacrificed after 96 hours for the usual tissue analyses. Table III shows the values obtained for the excretion and femur shaft. As expected, the 12 days' depletion caused a marked effect: at 24 days the main change was in the mineral content of the bone. After 24 days depletion there was no further demonstrable effect. These values considered in combination with those of Table II emphasize the finding that when a normal rat is placed on a low calcium diet, sometime between 4 and 12 days the bones increase in their capacity to accumulate absorbed Calcium 45 and this increased capacity remains fairly constant even though the bones lose considerable mineral through further depletion. It should be noted in consideration of these data that effects due to age in addition to depletion may have been a contributing factor.

Effects of specific activity at absorption site. The specific activity of the calcium available for a given study will determine the mass of calcium which must be administered to an animal to provide sufficient radioactivity for the measurements. It seemed desirable therefore to determine the mass level below which significant effects due to carrier calcium would not be encountered on both the normal and low dietary intake. This information is also necessary for interpretation of data on the factors governing absorption from the tract. In this study 2 groups of litter mate rats, reared as previously described on low and normal calcium rations were dosed orally with the same amount of Ca^{45} activity to which varying amounts of carrier were added as indicated in Table IV.

The results in Table IV indicated that the mass of calcium in the dose had little effect on the absorption of this element. With the exception of an increase in fecal excretion when 20 and 51 mg of calcium were given to the low calcium animals, there was no demonstrable effect. Analysis of tissue samples from these animals also showed no differences due to the mass of carrier calcium used. This is in agreement with the findings previously discussed. A preliminary report to this

TABLE IV. Effects of Mass of Calcium on Excretion of Ca^{45} Orally Administered to Rats on Low and Normal Dietary Calcium Levels (Rats Were 84 Days of Age).

Ration	No. of rats	Dose, mg of Ca	Fecal excretion of Ca^{45}, % of dose
Low Ca	3	.28	.50
	4	1.3	.60
	3	10	.85
	4	20	4.4
	2	51	3
Normal Ca	3	.28	31
	3	1.3	33
	3	10	40
	3	20	33
	3	51	26

effect was published by Hansard *et al.*(7) and essentially confirmed by Carlson(8).

Effects of age. The following study was designed to show quantitatively the effects of age of the animal on Calcium 45 absorption and accumulation in the bone. Four lots of weanling litter mate rats were placed on a normal (Ca-3) ration and at the intervals designated in Table V were given a single oral dose of Calcium 45 and sacrificed after 96 hours for the usual studies. As may be noted from Table V the younger rats excreted less of the Ca^{45} although there was no significant difference between the 6½ and 16-month-old animals in this respect. The calcium and ash content of the femur showed a progressive increase with age, whereas the Ca^{45} accumulation and specific activity showed a progressive decrease with age. From the Ca^{45} accumulation per unit of Ca^{45} absorbed it can be noted that similar values were obtained for the first 3 groups, whereas the 16-month-old animals showed a significant decrease. This may be interpreted to mean that between 1 and 6½ months the capacity for the Ca^{45} to accumulate in the bone from the extracellular fluid was relatively constant, while at 16 months of age this capacity had decreased.

Discussion. It is generally accepted that Ca^{45} introduced into the extracellular fluid undergoes a very rapid exchange with the calcium in the bone(9-11). Without entering into the controversial issues of the relationships of exchange and growth or the mechanisms of retention of Ca^{45} in bone, it is an expected and an established fact that practically all of the Ca^{45} which was originally present in the serum will eventually be found in the bone, from which it has displaced an approximately equivalent amount of stable calcium. As equilibrium is approached, at about 72 hours after oral administration, the specific activity of the skeleton will approach that of the serum, and due to the difference in the calcium content of the two tissues there will be of the order of hundreds of times as much Ca^{45} in the skeleton as in the serum. In any comparative experiment, then, a higher uptake of orally administered Ca^{45} in the skeleton cannot be interpreted as indicating an increased capacity of the bone for uptake of calcium, but may well reflect simply the fact that more Calcium 45 was absorbed from the tract and was thus available for exchange. It is suggested that the bone concentration of Ca^{45} per unit of Ca^{45} absorbed may be an indication of the exchange potential of the bone.

This exchange reaction must also be taken into consideration in the interpretation of excretion data. Where there has been absorption of Ca^{45} from the tract and the subsequent exchange with stable calcium of the bone, the excreta will contain a higher ratio of stable to radioactive calcium in proportion to the degree of exchange which has occurred. This should not be interpreted as an indication of demineralization.

An illustration of how the results obtained may depend upon the strict standardization of experimental conditions is furnished by consideration of the Ca^{45} fecal excretions of the 1-month rats in Tables III and V which were 8.8 and 23% respectively. The only difference between these two groups was that the Table III rats were placed on a low calcium diet after dosage, whereas the others were continued on the normal diet.

Summary. (1) The fecal excretion of orally administered Ca^{45} is a sensitive indication of the calcium status of the rat; (2) placing normal rats on a low calcium diet for as little as 2 days greatly increased their ability to absorb Ca^{45} from the tract; (3) the specific activity of labeled calcium at the site of absorption had little effect upon its absorption; (4) in general, younger rats absorbed more Ca^{45} than did older ones although there was no significant difference between 6½ and 16-

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TABLE V. Effects of Age of Rat on Excretion and Bone Accumulation of Orally Administered Ca^{45}.

Age, mo	No. of rats	Avg body wt (g)	Fecal excretion of Ca^{45}, % of dose	Femur shaft				Ca^{45}
				Ca^{45}*	mg Ca/g	Specific† activity	% ash	Fraction Ca^{45} absorbed
1	9	36	23	.57	90	6.3	25	.74
3	12	171	33	.45	168	2.7	49	.67
6½	4	312	56	.30	195	1.5	50	.68
16	6	310	51	.18	210	.86	56	.37

* μg of labeled Ca per g fresh tissue based on dosage of 10 μg per 100 g body wt.
† μg of labeled Ca × 10^3 per g of total Ca based on above dosage.

month-old animals; (5) for meaningful studies of this type, interpretations must include consideration of exchange reactions, and care must be given to initial selection of diets and animals, preparation of animals for the experiment, and dietary management during the experimental period.

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Inhibition of Urinary 17-Ketosteroid Excretion Produced by "Benemid".* (19103)

Lytt I. Gardner, John F. Crigler, Jr., and Claude J. Migeon.
(Introduced by Walter Fleischmann.)

From the Harriet Lane Home, Johns Hopkins Hospital, and Department of Pediatrics, Johns Hopkins University Medical School.

Benzoic acid(1,2) and carinamide (4′-carboxyphenylmethanesulfonanilide)(3-6) are known to block the renal tubular secretion of penicillin and thus to enhance plasma penicillin concentrations. A new compound, Benemid,† p-(di-n-propylsulfamyl)-benzoic acid has also been found to increase the plasma concentrations of penicillin and para-aminosalicylic acid (PAS)(7-11). Only 2 g of Benemid per day are necessary to produce consistent elevation of plasma penicillin and PAS levels(7-11), whereas 6 to 24 g per day of carinamide were necessary to produce similar results(3-6).

Bissell and colleagues(12) and Ceresa and

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† The authors are indebted to Sharp and Dohme, Inc., Glenolden, Pa. for the Benemid used in this study.

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Rubino(13) observed that carinamide greatly inhibited the renal excretion of 17-ketosteroids. The latter authors found that the administration of less than 6 g per day to an adult failed to diminish 17-ketosteroid excretion. Because of the similarities in the physiological action of carinamide and Benemid, an investigation has been made of the effect of the latter drug on urinary excretion of 17-ketosteroids, 11-oxycorticosteroids (neutral reducing lipids) and chemically measured estrogens (fluorogenic phenols) in normal man.

Methods. Two healthy, adult males, L.G. (Subject No. 1), age 33 years, and C.M. (Subject No. 2), age 26 years, served as subjects. Diet was unrestricted. Serial 24-hour urine collections were made during control periods and when Benemid was given. Each specimen was preserved with 10 ml of 25% acetic acid. 17-ketosteroids were measured by the meta-dinitrobenzene reaction in ethanolic KOH(14). This was done on urine extracts prepared by acid hydrolysis and subsequently extracted with carbon tetrachloride and washed with 2.5 N NaOH. Spectrophotometric curves to measure dehydroisoandrosterone were made on aliquots of these same extracts by the method of Allen and colleagues(15). A Beckman spectrophotometer, model DU, was used, with cells of 10 mm light path. Neutral reducing lipids were measured by the reaction of phosphomolybdic acid with chloroform extracts which had been washed 3 times with 0.1 N NaOH and partitioned between benzene and water(16). It is believed that the 0.1 N NaOH wash removed from the urine any Benemid which might have interfered with the steroid determinations. Urinary estrogens were measured chemically by a slight modification of Jailer's method(17).

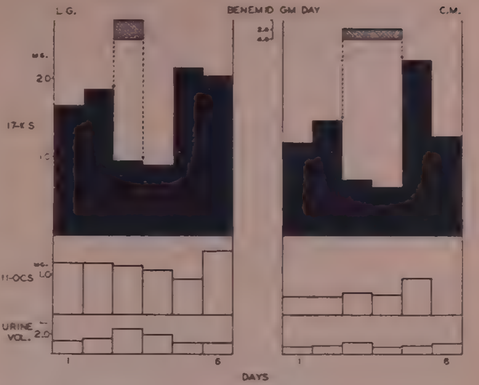

FIG. 1. Effect of oral Benemid administration on urinary 17-ketosteroids, 11-oxycorticosteroids and urine volume in two adults.

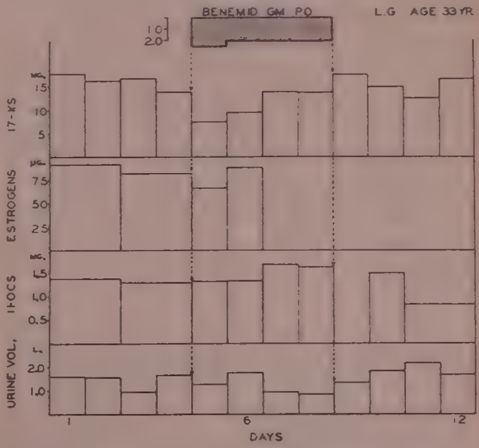

FIG. 2. Effect of oral Benemid administration on urinary 17-ketosteroids, estrogens, 11-oxycorticosteroids and urine volume in an adult.

Results. Fig. 1 shows the data obtained from a 6-day experiment on each of the 2 subjects. Administration of 4 g Benemid to subject No. 1 over a single 24-hour period (1 g each 6 hr) produced approximately 50% reduction in urinary 17-ketosteroid excretion. This reduction persisted for 48 hours. Subject No. 2 was given 2 g Benemid per day for 2 days (0.5 g each 6 hr). The relative diminution of urinary 17-ketosteroids was about as great as in Subject No. 1, but immediately upon withdrawal of the drug there was a return to normal. Benemid had no observable effect on the excretion of neutral reducing lipids in either subject.

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TABLE I. Effect of Oral Benemid on Urinary Excretion of Estrogens in Subject C. M.

Day of exp.	Benemid, g/day	Urine vol, ml/day	Urinary estrogens, μ/day
1	0	740	20.7
2	0	820	17.4
3	2	1050	15
4	2	750	12
5	0	810	27.2
6	0	1050	24

Fig. 2 presents information concerning a 12-day experiment on Subject No. 1. On the first day 2.5 g Benemid were given, followed by 2.0 g per day for 3 more days. There was an appreciable drop in urinary 17-ketosteroids on the first day of treatment. These values gradually rose during the remaining 4 days of treatment. Neutral reducing lipids showed no change on this regimen.

Urinary estrogen excretion has been adequately studied in only one subject receiving Benemid. Table I shows the diminution in urinary estrogen when Subject No. 2 was given 2 g of Benemid per day. Fig. 2 shows a few urinary estrogen values on Subject No. 1, but the data are insufficient for any conclusion.

Because of the possibility that the total 17-ketosteroid inhibition observed might involve retention of certain 17-ketosteroids and not others, a qualitative study by the method of Allen and colleagues was carried out on the neutral 17-ketosteroid urine residue(15). This spectrophotometric technique sharply distinguishes between androsterone and dehydroisoandrosterone.[‡] Fig. 3 shows the spectrophotometric absorption curves on urine extracts of Subject No. 1 studied with this reaction. The peak at 390 mμ may be produced by androsterone or related 3-alpha-hydroxyketones, whereas the peak at 600 mμ may be produced by dehydroisoandrosterone, one of the 3-beta-hydroxyketones. It is seen that there is no significant difference in configuration between the control curve and the Benemid curve.

Comment. It is of considerable interest to observe the very selective manner in which certain systems are inhibited by the renal blocking agents carinamide and Benemid. In previous studies it has been observed that Benemid increased plasma concentrations of para-aminosalicylic acid but had no effect on plasma concentration of the structurally similar compound, acetylsalicylic acid(8). The current observations concerning inhibition of urinary 17-ketosteroid excretion as contrasted with lack of effect on urinary neutral reducing lipids would support this principle.

In view of the above it would not be surprising to discover that the numerous 17-ketosteroid compounds measured by the Zimmerman reaction might be differentially influenced by Benemid administration. The spectrophotometric evidence here presented serves only to compare the excretion of androsterone and/or related steroids with the excretion of dehydroisoandrosterone and/or related steroids. There is no indication that this ratio has been altered by Benemid administration.

Summary and conclusions. (1) The data presented demonstrate that Benemid in doses of 2 g per day per adult produced approxi-

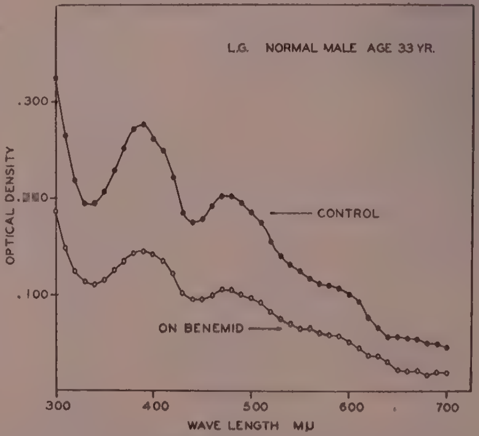

FIG. 3. Spectrophotometric absorption curves of color reaction for dehydroisoandrosterone done on urine extracts of subject L. G. Aliquots of .098 mg and .059 mg 17-ketosteroid were used for the upper and lower curves respectively in a final volume of 7 ml.

[‡] The authors are indebted to the Schering Corp. for crystalline androsterone and dehydroisoandrosterone acetate used as standards in this determination.

mately 50% diminution in urinary 17-ketosteroids. This diminution was not exaggerated by increasing the dose of Benemid to 4 g per day. (2) Spectrophotometric studies showed that Benemid did not change the androsterone-dehydroisoandrosterone ratio of urinary 17-ketosteroids. (3) Urinary estrogens were diminished in one subject. In another subject this diminution was questionable. (4) Urinary 11-oxycorticosteroids (neutral reducing lipids) did not appear to be affected by Benemid.

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Microbiological Assay Method for Thiamine Using *Lactobacillus fermenti* 36 as Test Organism.* (19104)

S. C. FANG AND JOSEPH S. BUTTS.

From the Department of Agricultural Chemistry, Oregon Agricultural Experiment Station, Oregon State College, Corvallis.

Lactobacillus fermenti 36 has been proposed by Sarett and Cheldelin, and Cheldelin *et al.* for thiamine assay of foodstuffs(1,2). In studies with this method, it was observed that a striking stimulation in growth resulted when filbert nut meal extract was added to the assay medium containing either a suboptimum or an optimum amount of thiamine. Growth stimulants have been shown to exist in certain foodstuffs for the growth of *L. casei* in the microbiological assays for riboflavin and pantothenic acid when a suboptimum amount of vitamin is present in the assay medium by Bauernfeind *et al.*(3). Chu and Williams(4) and Pollack and Lindner(5) also reported the existence of stimulatory factors for *L. casei.* Wright and Skeggs(6) and Colio and Babb(7) have demonstrated the existence of a stimulatory growth factor in trypsinized vitamin free casein and malt sprouts extract, respectively, for *Streptococcus lactis R.* In this laboratory a series of experiments showed that there are a number of substances which will stimulate the growth of *L. fermenti* 36 in the presence of any level of thiamine.

The experiments reported here demonstrate the stimulatory effects of maltose and takadiastase digested starch on the growth of *L. fermenti* 36 in thiamine assay medium. It also demonstrates that one may obtain an erroneous thiamine value by this method if the substances used are high in starch.

Experimental. In the microbiological assay of thiamine reported herein the procedure of Sarett and Cheldelin(1,2) was employed. The test organism used in this assay was *L. fermenti* 36. The extent of growth was determined quantitatively by measuring the turbidity after 16 hours of incubation at 37°C with a Klett-Summerson photo colorimeter, using a 54 filter. The readings are expressed in Klett units. In order to get a low blank value in this assay, a boiled alkali treated peptone was used in the medium. The

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TABLE I. Effect of Substances on Rate of Growth of *Lactobacillus fermenti* 36.

Substances added	Amt/10 ml, mg	Turbidity reading 0 µg B_1, Klett units	.1 µg B_1, Klett units	.5 µg B_1, Klett units
Standard thiamine		19	[illegible]	[illegible]
Yeast extract (Bacto product)	1		174	
	2		[illegible]	
Liver extract (Wilson's 1:20)	1		195	
	2		226	
Papain digested casein	1		178	
	2		182	
Ascorbic acid	.4		193	
	.8		210	
Maltose	2		174	
	4		[illegible]	
Papain and takadiastase digested filbert nut meal extract	2.5	210	210	
	5	223	230	
	10	254	246	
	15	274	268	
	20	290	283	
Takadiastase and papain	100 µg each	30	168	
	200 " "	36	175	

takadiastase digested starch was prepared as follows: 50 ml of boiling water was added to one gram of soluble starch; 40 ml of sodium acetate buffer (pH 4.5) and 20 mg of takadiastase were then added to the solution after it had cooled; the mixture was allowed to digest 24 hours at 37°C under a layer of benzene. It was then steamed for 30 minutes to remove all the benzene and to inactivate the enzyme, neutralized to pH 6.6-6.8, and adjusted to 100 ml with water and filtered through a dried filter.

Results. Effect of B vitamins. Although *L. fermenti* 36 grows fairly rapidly on the assay medium, it was found that the growth rate was increased appreciably when filbert nut meal extract was added to the assay tubes. Vitamins were added individually to the assay tubes in order to test whether the growth stimulatory effect of filbert nut meal extract could be replaced by some members of the B complex.† It was observed that no significant change in growth over that in the blank occurred except in the cases of nicotinic acid and pyridoxine, where slight stimulation occurred. Moreover, when the vitamins in the medium were increased 5 to 10 times, no increase in growth was observed. This indicated the sufficiency of the basal medium in this respect, and the stimulatory effect of filbert nut meal was not due to the known B vitamins tested.

Effect of other known compounds. Since the growth rate of *L. fermenti* 36 could be increased by filbert meal extract, it appeared advisable to test the possible effect of an additional series of known compounds.‡ The results are summarized in Table I.

Since the sample for thiamine assay in this method is prepared by digesting the substance with a mixture of takadiastase and papain, it is likely that the starch will break down into dextrin and maltose. When these compounds were tested they were found to cause stimulation of *L. fermenti* 36 in restricted thiamine medium. An erroneous result of thiamine assay by this method would be expected if the material contains starch. Results in Tables II to IV demonstrate the stimulatory effects of maltose or takadiastase digested starch on the growth of *L. fermenti*

[†] 5 µg and 10µg of riboflavin, pantothenic acid, nicotinic acid, p-aminobenzoic acid, and pyridoxine, 0.1 µg and 0.2 µg of folic acid, 0.4 µg and 0.8 µg of biotin or 10 µg and 20µg of inositol.

[‡] 4 to 8 mg of untreated Bacto-peptone or boiled alkali treated peptone, 0.8 mg of *L*-arginine, *L*-glutamic acid or *DL*-tryptophan, 0.4 mg of inositol, L-aspargine, succinic acid or maleic acid, 0.1 mg of xanthine and 4 mg of starch had no effect. However, yeast extract, liver extract, ascorbic acid, papain digested vitamin free casein and maltose are found to be stimulatory. Takadiastase and papain had slight thiamine activity and stimulatory effect.

TABLE II. Stimulatory Effects of Maltose or Takadiastase Digested Starch on Growth of *Lactobacillus fermenti* 36 at Various Thiamine Levels.

Thiamine levels, µg	Control, Klett units	Turbidity reading: Enzyme digd. starch added, 20 mg/tube, Klett units	Turbidity reading: Maltose 1 added, 20 mg/tube,* Klett units	Turbidity reading: Maltose 2 added, 20 mg/tube,* Klett units
0 (not inoc.)	0	5	0	0
0	60	71	80	78
.01	100	119	122	115
.02	124	145	135	137
.03	135	163	171	166
.04	144	183	186	184
.05	151	197	200	196
.10	175	246	240	244
.20	177	260	264	262

* Maltose 1 source unknown, Maltose 2 is Eastman Kodak Co. product.

TABLE III. Stimulatory Effects of Various Amounts of Maltose or Takadiastase Digested Starch on Growth of *Lactobacillus fermenti* 36 at .1 µg Thiamine Level.

Enzyme digested starch added per tube, mg	Thiamine level (Klett units)		Maltose added per tube, mg	Thiamine level (Klett units)	
	None	.1 µg		None	.1 µg
0	60	179	0	60	179
10	65	228	10	69	216
20	67	236	20	71	240
30	79	242	30	71	252
40	78	240	40	71	258

TABLE IV. Recovery of Thiamine in A Synthetic Sample Containing Takadiastase Digested Starch (each value is average of 4 tubes).

Synthetic sample			Recovery	
Amt added, ml	B_1 content per tube, µg	Enzyme digested starch per tube, mg	Sarett & Cheldelin's medium (1,2), %	Same medium + 40 mg maltose per tube, %
1	.005	3.5	98.8	93
2	.010	7	124	93.2
3	.015	10.5	146	99.2
4	.020	14	156	102
5	.025	17.5	160	105
Avg			137	98.6

36 and the percent recovery of thiamine with this method if a sample contains enzyme digested starch. Since the thiamine assay medium contains Bacto-peptone which is known to be rich in strepogenin(5) it would seem that the stimulatory effect of maltose or enzyme digested starch on the growth of *L. fermenti* 36 is not due to this factor.

It is evident that neither maltose nor enzyme digested starch has a sparing action on thiamine but will greatly enhance the thiamine activity on the early growth of *L. fermenti* 36. In other experiments not reported in detail we have added both maltose and takadiastase digested starch in microbiological assay of other B vitamins. Stimulation was found in the growth of both *L. arabinosus* 17-5 in suboptimum amount of pantothenic acid when using Skeggs and Wright's(8) medium, and *Strep. faecalis R.* in suboptimum amount of pyridoxine when using Rabinowitz and Snell's(9) medium. The stimulation is increased with increasing amounts of maltose or enzyme digested starch in pantothenic acid assay. It was found that dextrins and fructose also have a stimulatory

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effect on the early growth of *L. fermenti* 36. In all cases, these substances do not affect the lactic acid production after 72 hours of incubation.

In the microbiological assay of B vitamins, it is frequently found that the growth of organisms may greatly be stimulated by the natural product. We are now inclined to believe that enzyme digested starch is one of the many stimulants which may be the decisive cause of erratic results obtained in assay of B vitamins of some natural products; thus addition of it to the assay medium is recommended.

Summary. (1) Maltose or takadiastase digested starch has been found to be one of the many growth stimulants for *L. fermenti* 36 in assay of thiamine. It is also stimulatory to *L. arabinosus* 17-5 and *Strep. faecalis R.* in assays of pantothenic acid and pyridoxine. (2) Evidence has been obtained to prove that a precaution should be made on thiamine assay of starchy substances using *L. fermenti* 36 as test organism. Addition of enzyme digested starch or maltose to the assay medium is recommended.

We wish to express our appreciation to Dr. Vernon H. Cheldelin for valuable suggestions in this study.

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Chemical Inhibition of Decapsulation in the Beta Hemolytic Streptococcus. (19105)

LAWRENCE M. WEINER AND C. V. SEASTONE.

From the Department of Medical Microbiology, University of Wisconsin Medical School, Madison

The following study represents an attempt to explain the loss of hyaluronic acid capsular material (HA) from beta hemolytic streptococci which occurs spontaneously in culture or in washed suspensions of young organisms. The problem has already received considerable attention(1,2,3). In the pneumococcus the disintegration of the capsule has been shown to be an enzymatic process(4) and it is possible that a similar mechanism might be involved in the streptococci. Since hyaluronidase brings about rapid decapsulation, it was necessary to consider this enzyme as a possible factor which might be operating under natural conditions. Studies by McClean(2) and Pike(5) suggest that certain capsulated strains of streptococci may produce hyaluronidase under special conditions, although it is probable that this is a result of dissociation(6). In the course of our study, extensive attempts to demonstrate an agent in cultures of various ages or in mechanically disrupted organisms which would accelerate the normal rate of capsular disintegration were unsuccessful; we were unable to duplicate the results of Morison(1) who found small amounts of such an agent released by organisms. Possibly the examination of other strains would have been successful. In these attempts the measurement of the rate of capsular loss was the same as that to be described below in connection with the demonstration of chemical inhibition of a hypothetical decapsulating enzyme which comprises the main part of this report.

Material and methods. The beta hemolytic streptococcus used in this work, strain 4, was a group C strain isolated from guinea pigs during a fatal outbreak of lymphadenitis(3). This organism was grown in a pH 7.6, 2% proteose-peptone broth containing 10% sterile horse serum, 1% dextrose, and 0.5% NaCl. Ten ml of such neutralized cultures were in-

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TABLE I. Compounds Found to be Effective in Retarding Decapsulation.

	Residual capsular polysaccharide after 2 hr at 37°C. % of total extracted by boiling		
Sodium fluoride .005 M (3 determinations)	62	76	75
Controls (distilled water)	32	34	32
Sodium azide .05 M	66		
Control	33		
Sodium arsenate 1 M	55		
Control	40		
Sodium citrate .025 M	65		
Control	34		

oculated into 90 ml of warm fresh broth and incubated at 37°C. In an actual test, cells from 4.0 ml of this broth culture were washed in distilled water and resuspended in equal amounts of the dilutions of the various chemical agents to be investigated. After 2½ hours' incubation in a 37°C waterbath, samples were centrifuged, cells washed in distilled water, and prepared for HA determination by the turbidimetric method previously described(3). Cells to be tested for residual cellular polysaccharide were resuspended in 2.0 ml of M/15 pH 7.0 phosphate buffer and boiled for 20 minutes to release capsular material. After centrifugation, 1.0 ml of the supernate was added to 5.0 ml of 0.5 M pH 4.2 acetate buffer. One ml of the dilute acidified serum reagent was then added and mixed immediately. After standing at room temperature for ½ hour, the turbidity was determined in a Klett-Duboscq photoelectric colorimeter, using various thicknesses of glass plates for comparison standards. Turbidities were converted into milligrams of hyaluronic acid by means of hyaluronic acid turbidity curves prepared with purified HA.

The following salts at the indicated concentrations were found to be ineffective in retarding decapsulation: barium chloride .00009 M to .0009 M; cadmium chloride .008 M to .08 M; lead nitrate .00005 M to .005 M; lithium chloride at .01M; manganese sulfate at .002 M; mercuric chloride at .0005 M; potassium bromide .005 M to .05 M; potassium iodide .005 M to .05 M; potassium periodate .00005 M to .005M; silver nitrate at .009 M; sodium arsenite at .05 M; sodium cyanide at .05 M; sodium oxalate at .005 M.

Table I shows the effects of fluoride, arsenate, citrate and azide ions, which caused the retention of at least 50% more cellular HA than the control cells suspended in distilled water. Since the retarding effect of fluoride on decapsulation appeared most pronounced, all further experiments were confined to this ion and a wider range of fluoride dilutions was tested. This experiment indicated that the retardation of decapsulation becomes less effective with lower concentrations of fluoride; optimal activity was obtained at a concentration of .005 M NaF. The retarding effect is not apparent in concentrations less than .00025 M NaF.

Studies were conducted to determine the duration of the fluoride ion inhibitory effect. Washed cells were resuspended in .005 M NaF and in distilled water as controls. After 2, 4, 8, 12 and 18 hours' incubation, test and control samples were examined for residual cell polysaccharide. Polysaccharide levels of the fluoride treated organisms remained high throughout the first 4 hours of incubation while the control samples showed a rapid loss. After 4 hours, however, the effect of the fluoride begins to diminish. By 8 hours all detectable amounts of polysaccharide have disappeared from the cells in the control sample. Fluoride-treated organisms tested at this same time still retained 50% of the total capsular material. At 12 hours small amounts of polysaccharide were still detected. Morison(1) reports in this connection that 0.5 to 1.0% formalin inhibits capsular disintegration up to 20 hours, an observation which we have confirmed. The optimum level is 0.25% formaldehyde assuming formalin to be 37%. It appears that the duration of this effect is considerably longer than that of fluoride.

When encapsulated streptococci are treated with hyaluronidase, the enzyme causes a rapid shedding of the capsular material. Since fluoride was seen to retard spontaneous decapsulation, experiments were conducted to determine whether fluoride would also inhibit the decapsulating activity of a streptococcal hyaluronidase. Washed cells from 4.0 ml of

TABLE II. The Effect of NaF on Streptococcal Hyaluronidase Activity.

Dilution of hyaluronidase	mg residual capsular polysaccharide after 2 hr at 37°C: Hyaluronidase alone	mg residual capsular polysaccharide after 2 hr at 37°C: Hyaluronidase + NaF .005 M
1:2000	0	0
1:8000	0	0
1:16000	.05	.09
1:32000	.05	.10
0	.05	.10

(Total polysaccharide extracted by boiling, .13)

a 5 hour broth culture of strain 4 were resuspended in distilled water containing various dilutions of a crude group C streptococcal hyaluronidase and incubated in a 37° water bath. At the same time, cells were also suspended in .005 M NaF containing the same dilutions of hyaluronidase used above. After 2½ hours' incubation the cells were prepared for polysaccharide determination. The results of this experiment are summarized in Table II, indicating that fluoride in no way inhibited the decapsulating activity of the group C hyaluronidase.

In view of the reported evidence that fluoride acts as an enzyme inhibitor, studies were made to determine the possible mechanism of its inhibitory activity in this instance. Some inhibitors act by combining with trace metal activators such as magnesium and calcium. If fluoride acts in this manner, it was felt that the addition of magnesium or calcium ions might block its effect. To test this possibility, varying concentrations of magnesium sulphate were added to washed cells suspended in .005 M NaF and incubated for 2½ hours. After incubation, the cells were washed and tested for residual capsular polysaccharide. It was found that the addition of magnesium ions to the test system in concentrations ranging between .05 M and .006 M resulted in an almost complete loss of the fluoride effect. With lower concentrations, the blocking action is not as pronounced. A similar experiment also indicates that calcium, like magnesium, is able to block the retarding effect of fluoride on decapsulation of strain 4. With calcium, however, greater concentrations are required to demonstrate blockage.

In addition to the inhibition of decapsulation by chemical means, the effect of temperature on the process was studied in the same manner. After exposure of young capsulated cells to various temperatures from 4°C to 98°C for 40 minutes in M/15 buffer at pH 7.0 the residual HA was determined. The results appear in Fig. 1 and indicate that the rate of loss of capsular material in relation to temperature change could be partly under the influence of an enzyme system in agreement with the conclusions drawn by Morison(1).

Discussion. Although we were unable to isolate a specific decapsulating agent from beta hemolytic streptococci, indirect evidence confirms the assumption that some enzyme is concerned. In view of the established fact that fluoride, azide and arsenate are enzyme inhibitors, it is felt that their action in delaying spontaneous decapsulation as reported here may be the result of enzyme inhibition. The failure of fluoride to affect streptococcal

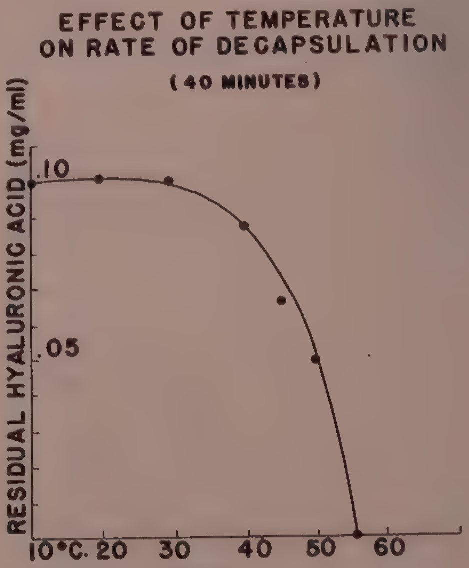

FIG. 1.

hyaluronidase, and the fact that all capsulated streptococci undergo spontaneous decapsulation even though they cannot be shown to produce hyaluronidase, would indicate that hyaluronidase is not responsible for this process.

In attempting to characterize the fluoride effect, it was found that the addition of magnesium or calcium to the test system tended to block the ability of fluoride to delay decapsulation. This may mean that fluoride acted in the system by combining with metallic ions necessary for enzyme action. However, MgF_2 and CaF_2 are highly insoluble compounds and it is felt that the observed blocking effect could also be due to the removal of available fluoride from the system.

Summary. (1) Attempts to demonstrate an agent in beta hemolytic streptococci which would accelerate the normal rate of capsular disintegration were unsuccessful. (2) Of various chemical agents tested, the following ions were ineffective in inhibiting this disintegration: Lead, cadmium, barium, manganese, lithium, silver, mercury, arsenite, iodide, bromide, periodate, cyanide and oxalate. (3) The following agents were found to retard decapsulation: Fluoride, azide, arsenate, citrate, and formaldehyde. The effect of fluoride was lost upon the addition of magnesium or calcium ions. (4) Fluoride did not inhibit the decapsulating action of streptococcal hyaluronidase. (5) The effect of temperature was found to be compatible with the assumption of a hypothetical enzyme system causing decapsulation.

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Action Mechanism of Cobra Venom, Cardiotoxin and Allied Substances on Muscle Contraction. (19106)

N. K. Sarkar. (Introduced by A. Szent-Györgyi.)

From the Institute for Muscle Research, Marine Biological Laboratory, Woods Hole, Mass.

The excised toad heart stops beating if perfused with a solution of cobra venom*, cardiotoxin†(1,2) or cardiac glucosides. The similarity of cobra venom to saponin was often noted by earlier workers, but later it has been shown, more closely, to resemble digitalis and digitalis-like substances(3,4). Now that a number of active principles of cobra venom have been isolated—some in a highly purified state—it was considered worthwhile to study the mode of action and to compare them with known cardiac drugs. Cardiotoxin, one of the many active constituents of cobra venom, has been isolated from it by fractional precipitation method(1). It is a protein and on weight basis it is 90-100 times more potent than crude venom. It contains less than 2% of neurotoxin and haemolysin.

Method. An isolated frog or toad heart (average weight of 250 g and 150 g respectively) was perfused with Ringer's solution. The minimum concentration of a drug required to stop the heart was taken as that amount which when perfused in the 5 cc of the test solution caused the systolic contracture. Test solutions were made up in salt mixture; the composition of which was as follows: NaCl, 6.5 g; $CaCl_2$, 0.12 g; $NaHCO_3$, 0.2 g; the total volume made up to 1000 cc. The pH was 7.2-7.4.

Results. Table I shows the concentration of each drug required for cardiac arrest. The

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* Cobra venom was extracted from the poison glands of cobra (*Naja tripudians*) and dried in a vacuum desiccator over fused calcium chloride.

† Cardiotoxin brings about the stoppage of perfused toad heart or heart beat when administered intravenously into an anaesthetized cat(2).

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TABLE I. Actions of Saponin, Digitoxin, Strophanthin, Cobra Venom and Cardiotoxin on Isolated Amphibian Heart.

		Condition of heart		
Substance	Min. conc. producing systolic arrest	(a) On perfusing with test solution	(b) After washing with Ringer's	Washing time, min
Cobra venom	1/500 for toad 1/1200 frog	Stopped in systole	Remained unchanged	40-60
Cardiotoxin	1/1050 toad 1/2500 frog	"	"	40-60
Saponin	1/1000 toad 1/1000 frog	"	Revived	20-30
Digitoxin	1/400 toad 1/1500 frog	"	"	20-25
Strophanthin	1/450 toad 1/2250 frog	"	"	50-60

last column of the table shows the time required to wash out the effect of each substance. From the table it will be also seen that the toad heart as a rule is more resistant to all drugs, except saponin, than is the frog heart.

It will be noted that the characteristic systolic contracture is caused by solutions of cobra venom, cardiotoxin, saponin, digitoxin and strophanthin. The cardiac arrest brought about by saponin and the drugs of the digitalis group can be completely reversed by washing; while the action of cobra venom and cardiotoxin cannot, even by repeated washing with Ringer. Among the other differences between these drugs, saponin has effects on red blood cells, the nervous system and the respiratory system, while cardiotoxin and the digitalis drugs lack such actions. Cobra venom too, has these properties of saponin, because it contains other active principles besides cardiotoxin.

Previously it was thought that calcium was involved in the development of systolic contracture of the perfused frog's heart caused by cobra venom(5). Without it the heart stopped in diastole. Similar results with digitalis drugs were obtained by Loewi(6). Our findings (Table II) indicate that calcium is not essential. When perfused with isotonic solution of NaCl, KCl or both together, the heart stops in diastole. When cobra venom or cardiotoxin is added to such a solution, the heart gradually revives and finally stops in systole and remains so even after prolonged washing with Ringer.

These results and the results obtained from the study of the action of cobra venom and cardiotoxin on the excitability and the contractility of nerves and muscles(7) led to the conclusion that they both act, perhaps, directly on muscle. To understand why the muscle contracts under the influence of cobra venom, the following experiments were performed.

An isolated muscle fibre from frog sartorius was placed in isotonic saline and 0.05 cc of cobra venom solution (1/2000-1/5000) was dropped on it. An extremely rapid contraction followed; amounting to 40-45% of the original length. Experiments using rabbit psoas muscle fibres were carried out in Petri-dishes containing 0.0015 M $MgCl_2$, 0.04 M veronal-acetate buffer of pH 7.0 and 0.1 M KCl. Cobra venom sufficient to make the final concentration to 1/1000-1/2000 when added, caused a contraction similar to that seen with frog muscle.

Muscle fibres in which the cell membrane has been removed by treatment with 50% glycerine solution no longer contract when cobra venom is added. These fibers were made according to the method of Szent-Györgyi(8). Thin strips of the musculus psoas of rabbit were treated with 50% glycerol, at 0°C for 24 hours and were kept for

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TABLE II. Influence of Different Ions on the Action of Cobra Venom and Cardiotoxin on Isolated Toad's Heart.

Substances in the perfusion fluid	Condition of heart on perfusion after washing— with the solution	with Ringer
a. Perfused with KCl	Stops in diastole	—
b. The same heart is then perfused with KCl and cobra venom	Heart revives and then stops in systole	Remained so
a. Perfused with NaCl, KCl	Heart stops in diastole	
b. The same heart is then perfused with NaCl, KCl and cobra venom	Heart revives and then stops in systole	" "
a. Perfused with KCl	Stops in diastole	
b. The same heart is then perfused with KCl and cardiotoxin	Heart revives and then stops in systole	" "
Cobra venom (alone)	Stops in systole	" "
Cardiotoxin (alone)	"	" "

4-5 days at –20°C in the same solution. It seemed of interest to investigate the possible effect of cobra venom on the recombination of actin and myosin in pyrophosphate treated glycerol-fibres(9); such fibres after treatment with cobra venom did not show any contraction on the addition of ATP over 0.26 M KCl. If association has been effected by cobra venom, one should expect contraction up to the concentration of .45 M KCl like glycerinated muscle fibres do.

Threads made of actomyosin prepared according to Weber(10) show no contraction on addition of cobra venom or cardiotoxin. Actin and myosin prepared according to the method of Straub(11) and Szent-Györgyi(12) still show superprecipitation on the addition of ATP and respective co-partner after treatment with cobra venom or cardiotoxin, indicating that these two muscle proteins are not affected directly by these compounds.

These results suggest that the pharmacological action of snake venoms requires the presence of the cell membrane, without which there will be hardly any contraction.

Summary. Cobra venom, cardiotoxin, saponin and drugs of the digitalis series bring about the stoppage of the movements of the excised heart. The cardiac arrest caused by cobra venom, unlike that by saponin and digitalis, is irreversible, *i.e.*, cannot be washed out. Unlike living muscle fibres, glycerinated muscle fibres and actomyosin threads show no contraction under the influence of cobra venom or cardiac drugs. These drugs have no effect on myosin and actin either.

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Specific Determination of Serum Creatinine.[*] (19107)

ZELMA MILLER[†] AND BENJAMIN F. MILLER.[‡]
From the Children's Medical Service, Massachusetts General Hospital, Boston

The estimation of serum creatinine by Jaffe's alkaline picrate reaction does not differentiate between creatinine and non-creatinine chromogen. This has led many workers

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[*] Aided by grants from the Research Corporation and the Bristol-Myers Co.

[†] Present address: Children's Cancer Research Foundation, Children's Medical Center, Boston.

[‡] Present address: Peter Bent Brigham Hospital, Boston.

to seek improved methods. Miller and Dubos developed an enzymatic method(1), based on the isolation of a soil microorganism capable of decomposing creatinine with a high degree of specificity(2). The difference between the Jaffe-reactive chromogen values before and after treatment with a suspension of the organisms represents true creatinine.

Borsook described a method for the determination of creatinine in tissues which involved separation of creatinine from other chromogenic compounds by adsorption on Lloyd's reagent(3). The results reported for tissues containing very high non-creatinine chromogen compared favorably with those obtained later by Baker and Miller, who employed the specific enzymatic method(4). Recently Hare adapted Borsook's method to the determination of creatinine in serum(5). A high degree of specificity was claimed, but this was not established by comparison with a specific method. To assess the specificity of the adsorption method, we have compared the enzymatic and adsorption methods on normal and uremic human serum.

Methods. We have obtained more consistent results with tungstic acid filtrates than with CCl_3COOH filtrates, such as Hare and Borsook employed. In all our determinations protein was precipitated by adding 3 ml of H_2O, 0.5 ml of 10% sodium tungstate, and 0.5 ml of 2/3 N H_2SO_4 for each ml of serum or plasma, according to Folin and Wu(6). The mixture was centrifuged for 10 minutes after standing at room temperature for 5 minutes with occasional shaking, and the supernatant was filtered through Whatman No. 42 paper.

Determination of creatinine by the adsorption method. Duplicate aliquots of this 1:5 filtrate, diluted, if necessary, to contain 5 to 15 μg of creatinine, are pipetted into conical centrifuge tubes and diluted to 4 ml. For normal sera, 4 ml of filtrate is a satisfactory volume. Glass-stoppered centrifuge tubes are preferable, but ordinary centrifuge tubes, sealed with Parafilm may be used. A 4 ml water blank and a creatinine standard containing 10 μg in 4 ml are set up in duplicate. Four-tenths ml of saturated oxalic acid and 30 to 40 mg Lloyd's reagent are added to each tube. The stoppered tubes are shaken vigorously 4 or 5 times during a period of 10 minutes and then centrifuged for 10 minutes. The clear supernatant fluid is aspirated carefully and discarded. In contrast to the determination in tissue, it is unnecessary to wash the Lloyd's reagent at this stage.

Creatinine is eluted by the addition of exactly 8 ml of alkaline picrate, prepared by mixing 6 parts of H_2O, 2 parts of approximately 0.04 M picric acid,§ and 2 parts of exactly 0.75 N NaOH. The Lloyd's reagent is dislodged from the bottom of the centrifuge tube and suspended in the alkaline picrate by vigorous shaking. The tubes are stoppered and allowed to stand for 10 minutes, during which they are shaken 4 or 5 times. They are again centrifuged for 10 minutes. The color intensity is determined in the Evelyn photoelectric colorimeter on 6 ml aliquots of clear supernatant, employing the 520 filter and the 6 ml aperture. Standards and unknowns are read against a reagent blank tube, set at 100% transmission.

Determination of specific creatinine by the enzymatic method. Total initial chromogen and chromogen remaining after treatment with bacterial enzyme suspension(7) are estimated by the Bonsnes and Taussky method(8) on 3 ml of a 1:5 tungstate filtrate containing 5 to 15 μg of creatinine. Specific creatinine represents the difference between these two

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§ This is prepared from picric acid containing approximately 10% water by dissolving 10.1 g in water and diluting to 1 liter.

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TABLE I. Comparison of Creatinine Values Obtained by the Enzymatic and Adsorption Methods.

Serum creatinine, mg %		
Apparent	Enzymatic	Adsorption
.67	.50	.51
.68	.49	.46
.74	.58	.49
.78	.56	.63
.85	.66	.67
.88	.71	.66
.90	.77	.68
.90	.78	.80
.94	.76	.72
.98	.76	.71
.99	.80	.76
.99	.84	.82
1.01	.75	.82
1.17	.95	.93
1.17	1.04	.95
1.19	.99	.99
1.21	1.05	.94
1.68	1.45	1.49
2.90	2.57	2.78
3.02	2.59	2.80
3.45	3.16	3.22
3.80	3.41	3.78
4.10	3.84	3.86
5.83	5.18	5.45
7.16	6.70	6.40
10	9.48	9.70
11.4	10.8	11
12.6	11.5	11.7
13.6	12.6	13.2
18.7	17.9	18

values.

Results. The results obtained with 30 serum samples are shown in Table I. There is good agreement between the true creatinine values obtained by the enzymatic and the adsorption methods. To demonstrate further that Lloyd's reagent adsorbs creatinine chromogen specifically, a sample of blood fairly high in residual chromogen was incubated with the bacterial enzyme until all the creatinine had been decomposed. The bacteria were centrifuged off and the supernatant fluid was treated with Lloyd's reagent in the usual manner. No non-creatinine chromogen was adsorbed.

A report has appeared from Newburgh's laboratory which indicates that the use of 1:4 tungstate filtrates of plasma or serum give considerably lower creatinine values than 1:10 filtrates(9). Even though our experiments were performed with 1:5 tungstate filtrates, it seemed desirable to check this finding. We determined the creatinine content of 6 normal human sera in 1:5 and 1:10 tungstate filtrates. The values for the 1:10 filtrate averaged 7% higher than the 1:5 filtrate (range, 3 to 13%). This is considerably less than the 21% average difference observed by Camara *et al.*(9), but still represents a significant discrepancy. We, therefore, recommend the use of 8 ml of 1:10 tungstate filtrate instead of 4 ml of 1:5 filtrate in the adsorption procedure. The volume of the blank tubes and standards should be adjusted accordingly. Otherwise, the details of the method are unchanged.

Conclusions. Serum creatinine can be determined specifically on tungstate filtrates by using either the Miller-Dubos creatinine-splitting enzyme, or by adsorption on Lloyd's reagent and subsequent elution by alkaline picrate.

The authors were greatly helped by Dr. Allan M. Butler in carrying through this study, and we express our thanks to him.

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Comparative Effects of Cortisone and 11-Deoxycorticosterone on Tissue Arginase Levels of Adrenalectomized Lactating Rats. (19108)

S. J. FOLLEY AND S. C. WATSON. (Introduced by Robert Gaunt.)
From the National Institute for Research in Dairying, University of Reading, England.

Arginase levels of rat liver(1-3), kidney (2,3) and mammary gland(2) are decreased

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after adrenalectomy. Pair-feeding experiments(4) have shown that this decrease is not due to post-operative anorexia and is therefore a more direct, perhaps a primary response to adrenal removal. Replacement experiments on adrenalectomized rats have given somewhat conflicting results. Fraenkel-Conrat *et al.*(1) found cortisone more active than 11-deoxycorticosterone acetate (DCA) in restoring the liver arginase levels. Folley and Greenbaum (2) obtained results suggesting that cortisone and 11-dehydrocorticosterone might be more effective than DCA in restoring both liver and mammary gland arginase levels, but later results from the same laboratory, published only in preliminary form(5), suggested that 11-dehydrocorticosterone was no more effective than DCA. In short-term experiments Kochakian and Vail(3) observed little or no restorative effect on liver arginase levels with cortical extract or DCA though both treatments partially restored the kidney arginase levels. The subsequent development of an improved technic for tissue arginase determination in our laboratory(6), coupled with the increased availability of cortisone have prompted a further study of the effect of corticoids on the arginase levels of liver, mammary gland, intestine and kidney in the lactating rat.

Methods. Uniparous Norway rats were adrenalectomized or sham-operated on the 4th day of lactation and killed as convenient on days 14-16. A group of adrenalectomized rats received 3.0 mg DCA in 0.5 ml arachis oil in 2 divided doses daily from the day of operation until the day before autopsy; this dose was chosen as being that which has so far been found to give the best maintenance of lactation in our rats after adrenalectomy (7). Cortisone (free alcohol) was administered to another group by subcutaneous implantation of 3 x 15 mg pellets on day 4; the mean total absorption (measured on 7 rats) was 1.22 ± 0.04 mg/day, thus providing a daily dose much larger than we have previously been able to administer to rats in experiments on tissue arginase levels(7). Arginase (fully activated with Mn^{++}) was determined on homogenates of liver, abdominal mammary gland, kidney and intestine as described by Folley and Greenbaum(4) for the first 3 tissues. In the case of intestine a 6-inch length measured from the pylorus was opened up, the contents carefully washed out, and the washed tissue homogenized into 29 volumes of Mn-saline, the incubation time in the enzyme determination being 10 min. As before, the milk retained in the mammary tissue was determined and the mammary arginase activities expressed on a "milk-free" basis.

Results. Data for body weight changes, organ weights and milk content of mammary tissue are given in Table I. The marked increase in weight during the lactation period of lactating rats receiving 3 mg DCA daily, previously observed(7), is confirmed. Adrenalectomy caused a decrease in weight of the liver and kidneys(4) which was fully reversed by DCA but hardly affected by cortisone.

The tissue arginase levels are given in Table II and the significance of differences between pairs of mean values are given in Table III. In connection with the probabilities given in Table III the relatively small numbers of rats in each group should be borne in mind. It is evident that adrenalectomy caused a significant decrease in the arginase activities of all 4 tissues and that cortisone was far more effective than DCA in restoring these levels towards the control values. In fact cortisone restored the arginase activities of liver, mammary gland and kidney to values which were not significantly different from those of the sham-operated rats, though we believe it possible that significance might have been attained had larger groups been available. On the other hand, while DCA exerted slight but statistically significant restorative effects on the mammary gland and kidney arginase it had no significant effect on the liver arginase. This last result contrasts

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TABLE I. Effect of Corticoids on Body Wt Changes, Wt of Liver and Kidneys, and Milk Content of Mammary Tissue of Rats Adrenalectomized on 4th Day of Lactation. Mean values with stand. errors are given.

Treatment	No. of rats	% change in body wt of mothers between 4th to 14th days of lactation	Mean body wt of mothers at autopsy, g	Mean wt of liver, g	Mean wt of kidneys, g	Mean % retained milk in abdominal mammary tissue
Sham operation	6	+ 5.3 ± .9*	252	12 ± .44	1.70 ± .04	62.5 ± 4.3
Adrenalectomy	6	— 3.1 ± 1.9†	216	8 ± .22	1.38 ± .03	41.4 ± 7.6
Adrenalectomy + cortisone	5	— 3.9 ± 1.9‡	221	9.3 ± .87	1.54 ± .07	59.4 ± 3.4
Adrenalectomy + DCA	5	+12.8 ± 2.6‡	271	12.1 ± .73	1.63 ± .08	57.4 ± 3.2

* Determined on 9 rats. † Determined on 11 rats. ‡ Determined on 7 rats.

TABLE II. Effect of Corticoids on Tissue Arginase Levels of Lactating Rats Adrenalectomized on 4th Day of Lactation. Mean values with standard errors are given.

	Tissue arginase [Folley & Greenbaum (6) units]					
	Liver		Mammary gland	Intestine	Kidney	
Treatment	Units/g moist tissue	Units/100 g rat	Units/g moist tissue	Units/g moist tissue	Units/g moist tissue	Units/100 g rat
Sham operation	1596 ± 167	7618 ± 753.5	219.8 ± 11.61	60 ± 3.96	57.1 ± 5.26	38.7 ± 3.77
Adrenalectomy	512 ± 42.6	1913 ± 157.1	19.8 ± 3.89	37 ± 3.65	24.6 ± 1.96	15.6 ± .92
Adrenalectomy + cortisone	1277 ± 144.6	5466 ± 920.3	189.6 ± 8.20	82.8 ± 7.15	50.2 ± 3.19	35.2 ± 3.08
Adrenalectomy + DCA	541 ± 80.7	2408 ± 372.8	94.5 ± 9.81	55 ± 3.02	36.5 ± 2.56	21.9 ± 1.55

TABLE III. Significance of Difference Between Pairs of Means.

Treatment comparisons		Liver		Mammary gland	Intestine	Kidney	
		Arginase units/g moist tissue	Arginase units/100 g rat	Arginase units/g moist tissue	Arginase units/g moist tissue	Arginase units/g moist tissue	Arginase units/100 g rat
Sham operation	v. adrenalectomy	‡	‡	‡	‡	‡	‡
	v. adrenalectomy + cortisone	*	*	*	†	*	*
	v. adrenalectomy + DCA	‡	‡	‡	*	‡	‡
Adrenalectomy	v. adrenalectomy + cortisone	‡	†	‡	‡	‡	‡
	v. adrenalectomy + DCA	*	*	‡	‡	‡	‡

* Not significant (P>.05). † Significant (P<.02). ‡ Significant (P<.01).

with previous findings from our laboratory (2,5) obtained with a different method of arginase activity assay. The results for the intestinal arginase are of interest. In this case the enzyme level was fully restored to normal by DCA while the cortisone treatment gave a significantly higher level than normal. It is, however, doubtful how much significance should be attached to the results since, in our experience, arginase determinations on intestine are more liable to error than the assays on the other tissues studied since we have found that the enzyme is very readily removed from intestinal tissue by the washing procedure necessary to free it from its contents.

In the sham-operated and adrenalectomized

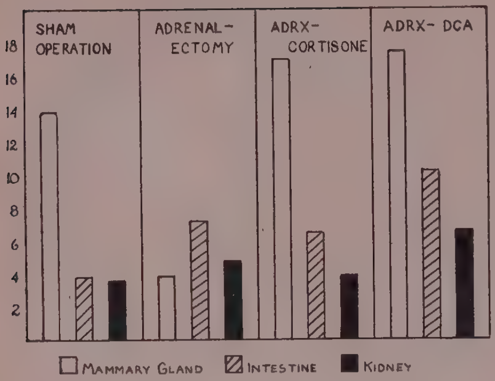

FIG. 1. Relative arginase activities (expressed per g moist tissue) of mammary gland, intestine and kidney as compared with that of liver, taken as 100.

groups the mean arginase activities of the mammary gland and kidney relative to the liver taken as 100 (Fig. 1), are very similar to the values previously found(4). As before, adrenalectomy profoundly altered the "spectrum" of relative arginase activities; neither steroid restored the distribution quite to normal but DCA gave much the more aberrant "spectrum".

Discussion. In this experiment cortisone was clearly more efficient than DCA in restoring the adrenalectomy-depleted arginase levels in all 4 tissues studied, a dose of approximately 1.22 mg/day of the former having in all cases a much greater effect than more than double this weight of deoxycorticosterone. The outcome is probably somewhat weighted in favour of cortisone by the fact that this corticoid was administered by what is probably the most efficient method, subcutaneous pellet implantation, while the deoxycorticosterone was given as acetate twice daily by subcutaneous injection in oil. Nevertheless it is extremely unlikely that the difference in effectiveness of the two steroids can be accounted for by the difference in method of administration. It is worth noting that although cortisone effected virtually complete substitution for the adrenals as regards the arginase levels of at any rate three of the four tissues studied, in other respects (body weight changes, organ weights) the cortisone-treated animals were clearly not normal. Moreover, by permission of Dr. A. T. Cowie who will report fully on this aspect in due course, it can be noted here that, in agreement with our previous results(7) and contrary to those of Gaunt *et al.*(8), cortisone in this experiment was no more effective than DCA in restoring lactation as measured by growth rate of litters, a finding mirrored by the values for the milk content of mammary tissue given in Table I. Both corticoids effected considerable, but neither effected complete, restoration of lactation.

Summary. Cortisone was much more effective than 11-deoxycorticosterone in restoring the depleted arginase levels of the liver, mammary gland, kidney and intestine in the lactating rat adrenalectomized on the 4th day of lactation.

We are greatly indebted to Dr. S. K. Kon for placing the facilities of his rat colony at our disposal; to Dr. A. T. Cowie for access to adrenalectomized lactating rats used by him in a lactation experiment; to Mr. C. P. Cox for statistical analysis of the results; to Dr. J. M. Carlisle of Merck & Co., Inc. for cortisone; to Dr. W. J. Tindall of Organon Laboratories Ltd. for 11-deoxycorticosterone acetate; and to Ciba Pharmaceutical Products Inc. for a grant to defray publication costs.

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3-Hydroxyanthranilic Acid Metabolism V. Distribution of Enzyme System in Animal Tissues.* (19109)

ROBERT E. PRIEST, ANN H. BOKMAN, AND B. S. SCHWEIGERT.

From the Division of Biochemistry and Nutrition, American Meat Institute Foundation and Department of Biochemistry, University of Chicago.

The enzymatic conversion of 3-hydroxyanthranilic acid to quinolinic acid is important from a nutritional standpoint, since it involves intermediate reactions in the metabolism of tryptophan to nicotinic acid. The chemical reactions are unique in that a benzenoid-type compound is transformed to a pyridine-type compound. *In vivo* experiments have shown that tryptophan can replace nicotinic acid in the diets of a variety of animals (rat, chick, dog, pig, horse, man, etc.). It has been shown that rat liver contains the enzyme system that metabolizes 3-hydroxyanthranilic acid to quinolinic acid(1-4) and that complete disappearance of 3-hydroxyanthranilate takes place when incubated with rat kidney slices(5). Other studies(6) have shown that an intermediate compound is formed in the metabolism of 3-hydroxyanthranilic acid to quinolinic acid by acetone-powder preparations or homogenates of rat liver. In order to obtain further information on the enzyme system in rat tissues, and for tissues available in larger quantities than rat liver, a study was made of the distribution of this enzyme system in various tissues of the rat and in pork and beef liver and kidney.

Material and methods. The rat tissues to be tested for the presence of enzyme were obtained from adult albino rats by decapitating the animal, removing the tissues desired, and using them immediately. The beef and pork tissues were frozen within one-half hour after removal from the animal and kept frozen until used. Samples were homogenized as described earlier(3) and diluted appropriately with M/10 phosphate buffer, pH 7.0, centrifuged in a clinical centrifuge at 1700 rpm (Cal. 193G) and the supernatant liquid used as the enzyme source. The day an assay was to be made 3-hydroxyanthranilic acid was dissolved in phosphate buffer, diluted to 50 μg/ml and refrigerated prior to use. After incubating the enzyme preparation and substrate for 30 minutes at 37°C, the enzyme was heat inactivated, the reaction mixture filtered, and aliquots taken for assay. The substrate was assayed fluorimetrically(3), and the product, quinolinic acid, determined microbiologically after decarboxylation to nicotinic acid. *Lactobacillus arabinosus* 17-5 was used as the test organism. Suitable enzyme and substrate blanks were included in each experiment. Rat liver was used as the basis of comparison and the composite data for the enzyme activity of rat, beef, and pork liver are presented in Table I. Comparable data for rat, beef, and pork kidney are shown in Table II. Three or more samples of each tissue with one or more levels of tissue were used in these studies. Acetone-powder preparations(3) were also made of rat and pork liver and beef kidney. These preparations showed the same activity as the homogenates on a dry weight basis.

Results and discussion. Homogenates of rat liver quantitatively converted 3-hydroxyanthranilic acid to quinolinic acid. From

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TABLE I. Effectiveness of Liver Homogenates in Converting 3-Hydroxyanthranilic Acid to Quinolinic Acid.*

Liver†	Enzyme conc., mg wet wt of tissue	Substrate metabolized, micromoles	Quinolinic acid found, micromoles	% conversion
Rat	10	.37	.39	105
	50	.65	.59	91
Pork	10	.31	.33	106
	50	.61	.52	86
Beef	10	.24	.20	81
	50	.65	.37	56

* .65 micromoles 3-hydroxyanthranilic acid, homogenate and buffer added to a total volume of 10 ml, and incubated at 37° for 30 min.

† Dr. L. M. Henderson and associates (private communication) have also observed that pork and lamb liver are essentially equal in enzyme activity to rat liver in the metabolism of 3-hydroxyanthranilate to quinolinic acid.

TABLE II. Effectiveness of Kidney Homogenates in Converting 3-Hydroxyanthranilic Acid to Quinolinic Acid.*

	Enzyme conc., mg wet wt of tissue	Substrate metabolized, micromoles	Quinolinic acid found, micromoles	% conversion
Rat	10	.21	.15	71
	50	.65	.13	20
	100	.65	.26	40
Beef	20	.35	.24	67
	100	.65	.25	38
Pork	20	.37	.29	78
	100	.64	.33	52

* See footnote, Table I.

91% to 105% of the substrate metabolized by rat liver homogenates and from 75% to 108% of the substrate metabolized by pork liver homogenates could be assayed as quinolinic acid (molar basis). Certainly it can be said that quinolinic acid is the primary product of this enzyme system. While beef liver was equally active in metabolizing 3-hydroxyanthranilic acid, all of the substrate metabolized could not be assayed as quinolinic acid. With the use of 10 mg of tissue about 80% of the substrate metabolized could be accounted for by quinolinic acid assay and with 50 mg of tissue only about 55% was found.

Homogenates of rat, pork, and beef kidney possessed equal activity per unit weight of tissue for metabolizing 3-hydroxyanthranilate (Table II) although only about one-half that observed for liver homogenates. When 10 or 20 mg of tissue were used, a large percentage of the substrate utilized appeared as quinolinic acid, but with 50 or 100 mg of tissue a much smaller percentage conversion was found. The micromoles converted to quinolinic acid remained approximately the same in either case. These results suggest that another primary product of 3-hydroxyanthranilic acid metabolism was formed. Since it is known that an intermediate compound is involved in the conversion of 3-hydroxyanthranilic acid to quinolinic acid(6), it seemed possible that this intermediate had accumulated at the end of the incubation period and could account for the low conversion to quinolinic acid. Kidney homogenates and beef liver homogenates were tested spectrophotometrically(6) for enzyme activity and evidence was obtained that although the intermediate compound was formed with these enzyme preparations, it was also rapidly broken down as shown by a reduction in the optical density at 360 mμ indicating that this intermediate was not an end product of the reaction.

Possibilities to explain these findings include the further transformation of part of the quinolinic acid after it is produced and the metabolism of a portion of the substrate by alternative pathways which may or may not include the intermediate. An experiment was conducted with the addition of quinolinic acid to beef liver and kidney homogenates and no evidence for the metabolism of the added quinolinic acid was observed.

Several other tissues of the rat were tested and found not to metabolize 3-hydroxyanthranilic acid to quinolinic acid in measurable amounts at the enzyme concentrations used (10-100 mg wet weight of tissue). Muscle, spleen, pancreas, brain and testis homogenates all metabolized small but significant amounts of substrate, but in no case were detectable amounts of quinolinic acid produced, and no spectrophotometric evidence for the formation of the intermediate compound was obtained.

Summary. A survey of various tissues of the rat, and of beef and pork liver and kidney was made for the presence of the enzyme system that converts 3-hydroxyanthranilic acid to quinolinic acid. It was found that rat and pork liver homogenates quantitatively convert 3-hydroxyanthranilic acid to quinolinic acid. Beef liver also contains this enzyme system, but not all of the substrate metabolized could be accounted for as quinolinic acid. Rat, pork and beef kidney likewise contain the enzyme system but again not all of the 3-hydroxyanthranilic acid metabolized was found by the quinolinic acid assay. Spleen, muscle, brain, pancreas and testis of rats either do not contain the enzyme system or contain it in very small quantities. No evidence was obtained to indicate that the intermediate compound previously described had accumulated to account for the lower production of quinolinic acid by these preparations.

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Glucose Formation by Kidneys in Eviscerated Dogs.* (19110)

BRUCE MACKLER, PETER AMMENTORP,† HARRY GRAUBARTH, AND GEORGE M. GUEST.
From the Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati, Cincinnati, O.

Several investigators have offered evidence that the kidneys may be a source of blood-sugar(1-6). Cohn and Kolinsky(6) reported evidence of glucose production by the kidneys, finding the rate of disappearance of glucose from the blood of hepatectomized and nephrectomized dogs nearly double the rate found in dogs hepatectomized without nephrectomy. In experiments here reported, (designed for further study of this question), evisceration was done, with and without nephrectomy, to eliminate possible effects of insulin production on the rate of disappearance of sugar from the blood in hepatectomized dogs.

Procedures. Ten mongrel male and female dogs, previously fasted 3 days with water allowed *ad libitum,* were eviscerated by the method of Markowitz(7) under nembutal anesthesia. Five dogs were bilaterally nephrectomized with the evisceration and in 5 the kidneys were left intact. Samples of venous blood were drawn for analysis immediately following operation and at 15-minute intervals during the next hour. Blood-sugar was de-

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† Visiting Research Associate, from Copenhagen, Denmark.

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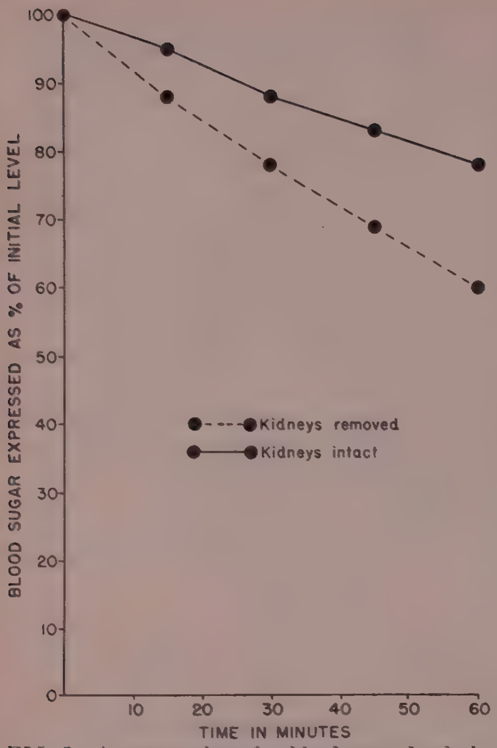

FIG. 1. Average values for blood-sugar levels in 5 dogs after evisceration with kidneys intact and in 5 dogs eviscerated and nephrectomized.

termined in duplicate by the Nelson modification(8) of the Somogyi method.

Results. Graphs in Fig. 1 represent averages of the concentrations of blood-sugar at the intervals indicated, expressed as per cent of initial levels(9). The average rate of disappearance of glucose from the blood in the nephrectomized dogs was nearly double that observed in dogs with intact kidneys. Statistical tests, by the method of analysis of variance(10), indicated a highly significant difference ($p < .01$) between the disappearance rates.

Discussion. Results of these experiments are in accord with the findings of Cohn and Kolinsky and furnish supplemental evidence that the presence of the pancreas was not a factor influencing the blood sugar disappearance rate in hepatectomized dogs. The data indicate that in hepatectomized dogs the kidneys may produce an amount of glucose equivalent to half that which disappears from the blood immediately after operation.

Summary. In 5 dogs subjected to evisceration and bilateral nephrectomy the rate of disappearance of sugar from the blood immediately after operation was nearly double the rate observed in 5 dogs similarly eviscerated but not nephrectomized, indicating that in hepatectomized dogs the kidneys may be an important source of sugar supplied to the blood stream.

TABLE I. Disappearance of Blood-Sugar in Dogs After Evisceration, With and Without Bilateral Nephrectomy.

Dog No.	Preparation	Blood-sugar following evisceration, mg/100 cc 0 min	15 min	30 min	45 min	60 min
1	Evisceration with kidneys intact	57	53	49	44	37
2		135	127	120	116	112
3		82	77	72	68	68
4		91	88	79	75	70
5		261	254	237	229	217
6	Evisceration with kidneys removed	96	83	71	66	55
7		58	50	42	31	25
8		93	79	71	65	58
9		73	64	59	52	46
10		81	78	69	66	60

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Accumulation of Radioiodine (I^{131}) in Thyroid Gland of the Hamster Embryo.* (19111)

Louis A. Hansborough and Hagalyn Seay. (Introduced by Arnold Maloney.)
From the Department of Zoology, Howard University, Washington, D. C.

The correlation between the storage of colloid and functional activity has been the basis upon which many of the studies on the thyroid gland have been made both in the clinic and in the laboratory. This relation is also the basis upon which its initial function in the embryo can be established. Studies of this type are facilitated where radioactive iodine (I^{131}) is used as a tracer, since its presence in the colloid is easily demonstrated on a photographic emulsion. Recently, with the use of radioactive iodine, Hansborough and Khan(5) demonstrated that the thyroid gland of the chick embryo becomes functional toward the end of the 11th day of incubation and that the activity of the gland increases with the increase in the number of its follicles. Gorbman and Evans(4) found that radioactive iodine appeared in the colloid of the frog thyroid when the young tadpole reaches a length of 10 mm and also that the storage of colloid is correlated with the appearance of the thyroid follicles. These workers(3) also determined the age at which the thyroid of the fetal rat begins to function. They studied 20 fetuses of rats in the 15th to the 21st day of gestation. Of those studied, thyroids of none showed evidence of iodine storage before the 19th day of development. Coincident with the beginning of function of the thyroid in the 18-19-day-old rats, follicle differentiation occurred. In the human fetus, it has been observed(1) that the accumulation of radioactive iodine and the differentiation of follicles in the thyroid occur from the 14th to the 32nd week of development.

The purpose of this study is to establish the initial up-take of radioactive iodine by the thyroid of the embryo hamster and thus to reveal the age of the embryo at which the thyroid becomes functional.

Materials and methods. Forty females were mated to 7 males (an average of 6 females to each male) and the time of copulation was recorded as the beginning of development, since it has been established that ovulation and fertilization follow within a few hours after mating(2). After copulation, the females were weighed and isolated in individual cages. Increase in weight was used as an index of pregnancy, particularly in the early stages of development, when observable size increase was negligible. With a No. 2 tuberculin syringe, the females of 8 to 15 days gestation (gestation period is 15 days 8 hours) were injected intraperitoneally with a solution of radioactive iodine (25 microcuries/cc) of from 50 to 100 microcuries, the amount of fluid not exceeding 2 cc per injection. A period of from 15-30 minutes was allowed after the last injection before the females were sacrificed and the embryos recovered. This was suggested by the work of Hertz, *et al.*(6) who found that the normal or hypoplastic thyroid of the rabbit collected a maximum of radioactive iodine within 15 minutes after injection and that for longer periods the amount collected was not greatly increased. The activity of the embryos was measured with the Geiger-Muller counter and whole embryos were fixed according to the method previously described(7). Similarly the ac-

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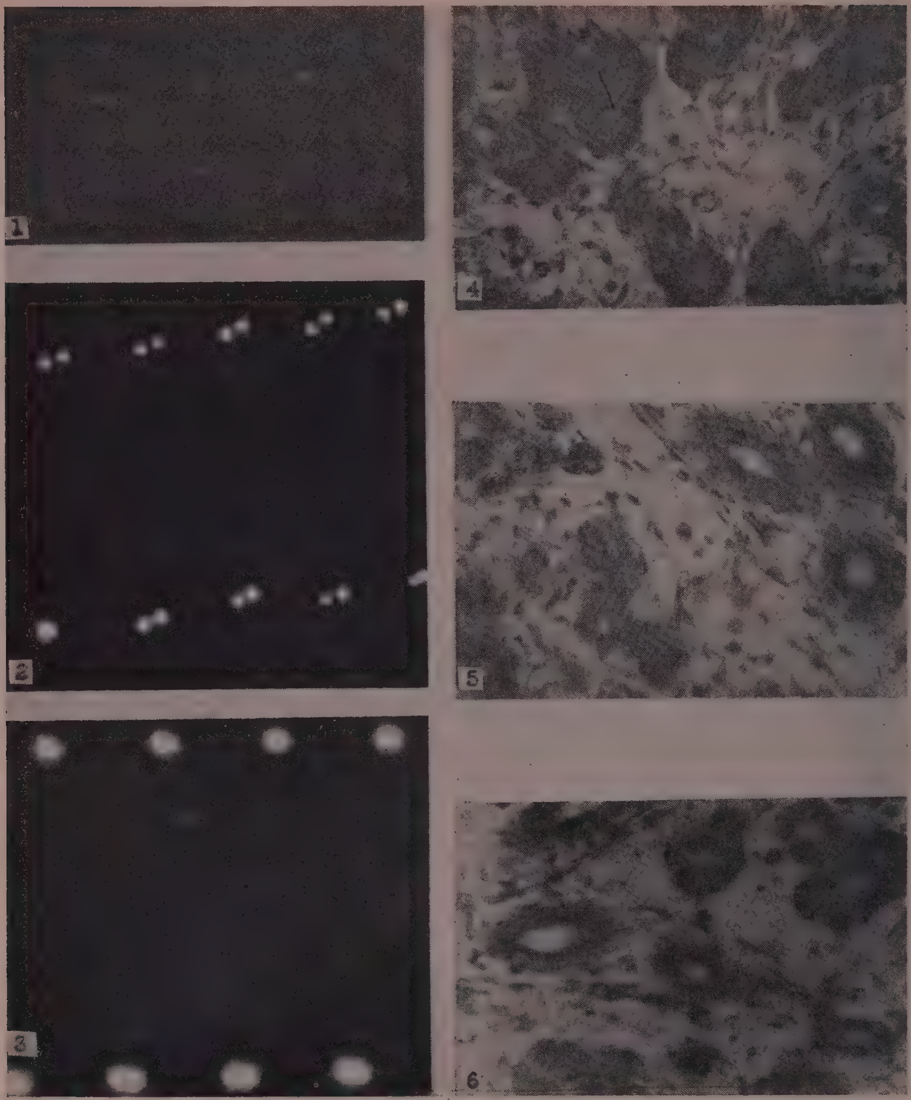

FIG. 1. 1. Autoradiograph of sections through the thyroid of a 13-day embryo. 2. Autoradiograph of sections through the thyroid of a 14-day embryo. 3. Autoradiograph of sections through the thyroid of a 15-day embryo. 4. Microphotograph of the sections shown in 1. Note small follicles surrounded by vascular connective tissue. 5. Microphotograph of the sections shown in 2. 6. Microphotograph of the sections shown in 3. Note the increase in size and number of the follicles shown in 5 and 6.

tivity of the thyroids of the mothers was checked. Dental x-ray films were exposed to sectioned embryos for from 6 to 8 days, depending upon the activity of the iodine. After autoradiographs were made, sections were stained in Delafield's hematoxylin and eosin for microscopic study.

Observations and conclusions. Autoradiographs of embryos in the 8th to the 12th day of development showed no evidence of iodine accumulation. The first indication of the collection of radioactive iodine was observed in autoradiographs of 13-day embryos. Further study showed that iodine accumulation increased with the age of the embryo, as indicated by the increase in blackening on the x-ray film. These observations were supported by measurements with the Geiger-Muller counter; embryos of 8 to 12 days development gave no significant measurements. However, those of 13 days and older gave an increasing number of counts.

Microscopic study of sections of the thyroid of embryos from the 9th and 10th day of development revealed the presence of epithelial cells in irregular cords, but no discrete follicles. Thyroids of embryos of the 11th and 12th day contained a few groups of cells arranged in circular patches (young follicles), with a few discrete follicles in the 12-day embryos. There is a progressive increase in the number of follicles from the 12th through the 15th day of development with an increase in the quantity of colloid present.

Since it is assumed by many workers that the storage of colloid in the follicles indicates the function of the thyroid, then we may also assume that the initial appearance of colloid indicates the onset of thyroid function. If these assumptions are correct, then our observations support the conclusion that the thyroid of the embryo hamster begins to function on the 13th day of development, a short time after the first appearance of follicles.

It has been demonstrated for the embryos of many animals, including the human fetus (see introduction) that the storage of colloid is correlated with the differentiation of follicles in the gland, the quantity depending upon the degree of differentiation and the number of follicles present and these, in turn, are correlated with the age of the embryo. Our findings through autoradiographic and microscopic study support these conclusions for the thyroid gland of the golden hamster.

Summary. (1) Fifty to 100 microcuries of radioactive iodine in the form of NaI were injected into the peritoneal cavity of the gestating hamster on the 8th to the 15th day after copulation. (2) Observations made on embryos of from 8 to 15 days of development by means of autoradiographic study and tests with the Geiger-Muller counter show that radioactive iodine begins to accumulate in the thyroid on the 13th day of development. (3) The accumulation of radioactive iodine is coincident with the appearance of discrete follicles, which first appear between the 12th and 13th day of development. (4) The quantity of iodine-containing colloid increases with the number of follicles present and the age of the embryo.

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Cross-Immunity Tests in Animals with Epidemic Keratoconjunctivitis and St. Louis Encephalitis Viruses. (19112)

ISAAC RUCHMAN.
From Department of Bacteriology, College of Medicine, University of Cincinnati and the Cincinnati General Hospital.

Recent investigations disclosed that it is impossible to differentiate the viruses of epidemic keratoconjunctivitis (EK) and St. Louis encephalitis (SLE) by serological means(1,2). A hint that the two viruses may be different comes from the fact that EK caused a fatal encephalitis in guinea pigs and rabbits while SLE engendered only a nonfatal pyrexia in guinea pigs and nothing in rabbits. However, no distinct differences could be demonstrated by cross-immunity tests in animals. This report presents the data on the infectivity tests as well as the results of the cross-immunity tests.

Materials and methods. Viruses. The 2 strains of epidemic keratoconjunctivitis virus

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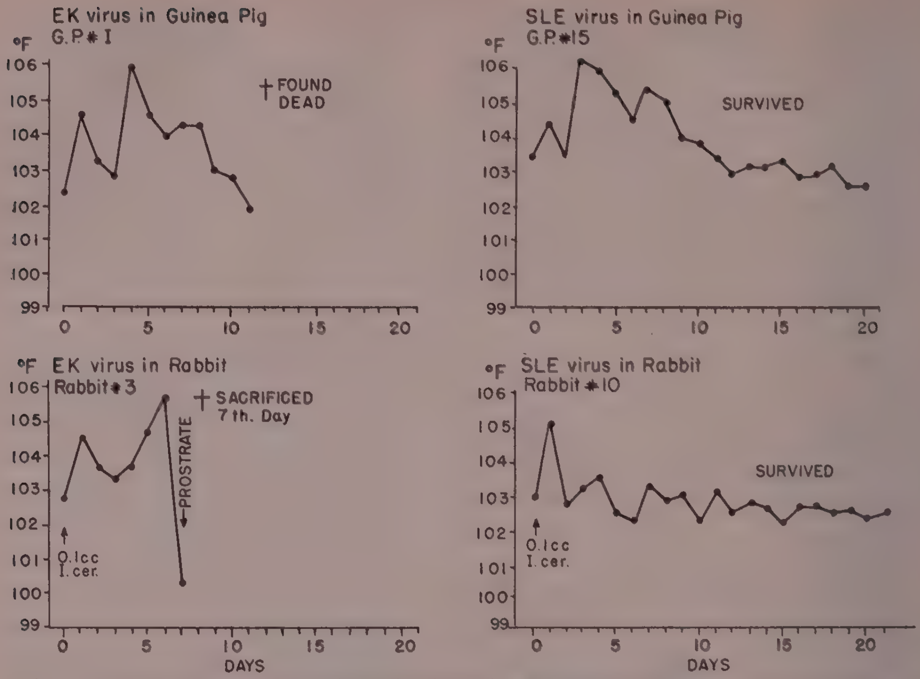

FIG. 1. Temperature curves. Rabbits and guinea pigs inoculated intracerebrally with St. Louis encephalitis and epidemic keratoconjunctivitis virus.

used in these tests were designated EK-103 and EK-TC17. They both came from Dr. A. E. Braley and were originally derived from the New York strain isolated by Dr. M. Sanders(3). Two strains of St. Louis encephalitis virus were used. One obtained from Dr. A. B. Sabin was designated *Webster* and the other supplied by Dr. W. McD. Hammon was designated *Hammon*. The Nakayama strain of Japanese B encephalitis virus (Jap B) was obtained from Dr. Sabin and the West Nile encephalitis virus (W. Nile) was supplied by Dr. Joel Warren. *Virus suspensions*. The virus suspensions were prepared by grinding infected mouse brains in a mortar with alundum and enough undiluted, normal rabbit serum to make a 20% suspension. After light centrifugation the supernatant fluid was distributed in 1 cc rubber stoppered vials and stored in dry ice. Virus dilutions were made in saline containing 10% normal rabbit serum. Although some variation occurred viruses used in this study generally titred 10^{-8} or higher. In a few instances 30% suspensions of EK infected rabbit brain were used to infect rabbits. *Animals*. The mice were albinos of either sex and were bred in our own laboratory. Those used in preparing the virus suspensions were between 18 and 24 days old and weighed approximately 8 to 11 g. Those that were used in the immunity studies were 6 to 8 weeks old and weighed around 20 g. All other animals were procured locally. The guinea pigs were of both sexes and weighed between 300 and 700 g. Rabbits also were of either sex and weighed between 2 and 3 kg. Albino rats were young males weighing approximately 150 g. Animals inoculated intracerebrally received 0.1 cc of a 10% suspension. Mice were only given 0.03 cc. The amount inoculated subcutaneously in mice was 0.1 cc of the designated dilution. Ether anesthesia was used for all intracerebral inoculations. As previously reported, the EK and SLE viruses behaved differently in rabbits and guinea pigs, and characteristic tem-

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TABLE I. Summary of Results in Animals.

Animals	Initial inoculation Virus inoculated	Initial inoculation No. tested	Result Died	Result Survived	Test for immunity Virus inoculated	Test for immunity No. tested	Test for immunity No. immune
Guinea pigs	EK	12	10	2	SLE	2	2
	SLE	8	1*	7	EK	7	5†
Rabbits	EK	14	11	3	EK	1	1
	SLE	4	0	4	”	3	3
	Jap B	2	0	2	”	2	0
	West Nile	2	0	2	”	1	0
Rats	EK	6	0	6	—	—	—

* Died following cardiac puncture.
† Guinea pigs that died succumbed to intercurrent infection.

perature curves are shown in Fig. 1. Unless indicated otherwise the 103 strain of EK and the *Webster* strain of SLE were used in most of the tests.

Results. Several series of tests were performed and in most cases surviving controls were used for subsequent immunity tests. Results are shown in Table I. Twelve guinea pigs inoculated intracerebrally with the EK virus developed fever of several days' duration. Ten of them died between 7 and 28 days after injection. The 2 survivors were tested with SLE virus at a later date. They did not develop pyrexia and were therefore considered immune. Eight guinea pigs inoculated with SLE virus developed fever. One animal died following cardiac puncture 3 weeks after the initial inoculation and 7 survived. The 7 survivors were tested for cerebral resistance against the EK virus. Five survived and 2 died of an intercurrent infection. Thus animals immune to EK were also immune to SLE and conversely, animals resistant to SLE were also resistant to EK.

Fourteen rabbits injected intracerebrally with EK virus developed fever. Three inoculated with the TC17 strain died and 8 out of 11 inoculated with the 103 strain died. One of the 3 survivors was retested with the EK virus and it was found to be immune. It was impossible to test EK immune rabbits for immunity to SLE. Two rabbits each were inoculated intracerebrally with the *Webster* and *Hammon* strains of SLE. The animals remained afebrile. Three of the animals were tested with the EK virus at a subsequent date and they remained well. Two animals were available that had been hyperimmunized against Jap B and one was available that had been hyperimmunized against W. Nile. They had received numerous peripheral inoculations of living virus over a prolonged period of time. They were inoculated intracerebrally with EK and although they became febrile, they did not die. All 3 controls for this experiment developed fever but only one of them died. It thus becomes apparent that although rabbits immunized against SLE were resistant to the EK virus the same thing could not be demonstrated under the conditions of this experiment for rabbits immunized against Jap B and W. Nile viruses.

Three young adult white rats were inoculated intracerebrally with the TC17 strain of EK and 3 others were inoculated with the 103 strain. None of the animals showed any obvious signs of illness during the period of observation.

It was somewhat more difficult to get immune survivors among mice. Nevertheless some animals were obtained in the following manner. Twenty g mice were inoculated subcutaneously with 0.1 cc of a 10^{-2} dilution of virus. After a period of 2 to 3 weeks they were given 0.1 cc of a 10% suspension subcutaneously. Two weeks later the EK immunized mice and one month later the SLE immunized mice were inoculated intracerebrally with a 10^{-2} dilution of the homologous virus. After a waiting period of 5 weeks the survivors were tested for resistance against an intracerebral inoculation of a 10^{-2} dilution of the heterologous virus. The data appear in Table II. Seven mice that had survived a previous intracerebral injection of the EK virus also survived inoculation of the SLE

TABLE II. Intracerebral Cross-Immunity Tests in Mice.

Exp.	Tested with*	Previously immunized with†	Results in tested mice‡
1	SLE	EK	S,S,S,S,S,S,S
		Controls	3,3,3,4,4,4,4,5,5,5
2	EK	SLE	7,10,11,S,S,S
		Controls	2,3,4,4,4,4,4,4,5,5
3	Jap B	EK & SLE	4,4,7
		Controls	3,4,4,4,5,5,5,5
4	West Nile	EK & SLE	5,5,6,7
		Controls	4,4,4,5,5,5,6

* Test dose consisted of a 10^{-2} dilution prepared from an infected mouse brain suspension stored in solid carbon dioxide.

† Immunized mice had survived a previous intracer. challenge with a 1% suspension of the homologous strain of virus.

‡ Figure indicates day of death; S indicates survival.

virus. Conversely of 6 mice that were resistant to SLE, 3 were now resistant to the EK virus while 3 succumbed after prolonged incubation periods. Seven of the above survivors now immune to both EK and SLE were divided into 2 groups Three mice were examined for cerebral immunity to Jap B virus and 4 were tested against W. Nile virus. They all succumbed to a 10^{-2} dilution of virus inoculated intracerebrally.

Discussion. It was previously shown that by serological methods the viruses of epidemic keratoconjunctivitis and St. Louis encephalitis could not be distinguished(1,2). The present immunity results confirm the close relationship that exists between the 2 viruses and seemingly it is only by means of intracerebral inoculation in rabbits and guinea pigs that the viruses can be told apart. It is not apparent at the present time whether the results of the immunity tests in mice are of significance or whether they merely represent differences that might be found to occur among different strains of the same virus. The current results do not add anything new to previous reports but do confirm the remarkable similarities that exist between the viruses of epidemic keratoconjunctivitis and St. Louis encephalitis.

Summary. Intracerebral inoculation of epidemic keratoconjunctivitis virus caused a fatal encephalitis in rabbits and guinea pigs, whereas inoculation of St. Louis encephalitis virus caused a non-fatal pyrexia in guinea pigs and elicited no response in rabbits. Mice were equally susceptible to both viruses while rats were refractory. Guinea pigs recovered from EK infection were also immune to SLE and those animals recovered from SLE were immune to EK. Rabbits convalescent from SLE were immune to EK, but the reverse experiment could not be done. Although EK immune mice resisted SLE, the reverse was not completely true.

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Modification of Sensitivity to X Radiation by Morphine Sulfate.* (19113)

J. B. KAHN, JR. (Introduced by J. Furth.)
(With the technical assistance of J. J. Lane.)†
From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

Lowered oxygen tension has been shown to prevent damage from X radiation in a variety of organisms(1-4). Anoxia has been produced in mammals by various methods, and has been shown to protect the animals from radiation lethality to some extent. Bacq and Herve (5) produced histotoxic anoxia with cyanide before irradiation, and demonstrated increased survival in mice so treated. Storer and Coon (6) produced a high degree of methemoglobinemia in mice by injection of p-aminopropriophenone (PAPP), and demonstrated that protection against X rays resulted. This finding has been confirmed independently in this laboratory. Another method of reducing oxygenation of tissues is by the production of anoxic hypoxia, by reducing the amount of oxygen in the atmosphere as in the work of Dowdy *et al.*(3), or by depressing the respiratory center. Morphine is outstanding in its ability to depress the respiratory center. Therefore, a series of experiments was performed to determine whether the administration of morphine would protect animals against the lethal effects of X radiation.

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† We are indebted for statistical analyses to Dr. A. W. Kimball, Jr., of the Biometrics Section, ORNL Mathematics Panel.

Materials and methods. Animals used were ZBC strain mice,‡ 10 to 12 weeks old at the time of injection, and weighing between 25 and 30 g. Morphine sulfate was dissolved in sterile physiological saline in a concentration of 15 mg/ml; 0.1 ml of this was injected per 25 g mouse. Injections were made intramuscularly with sterile precautions 30 minutes before exposure to the radiation. At the time of exposure to the X rays all mice showed positive Straub tests, and analgesia was seen in decreased pain responses to pinching of the tail and feet. Factors of irradiation were: 250 kvp, 30 ma, TSD 93.7 cm, filtration 3 mm Al, inherent filtration 1 mm Al, HVL 0.4 mm Cu. The dose rate, measured with a 100 r Victoreen r-meter, was 56.5 r/min. Mice were exposed in groups of 20, individually compartmented in a Lucite box. The maximum variability in dose delivered to the various compartments was 2%. Controls were uninjected irradiated mice, since a preliminary experiment indicated that the injection of 0.1 ml of physiological saline had no effect on X-ray mortality. Figures reported are for 30-day mortality, as no deaths occurred after this period.

Results. A preliminary experiment was performed using morphine dosages of 24, 60, and 90 mg/kg, and X-ray doses of 800 and 900 r. The results indicated that enough protection was afforded by morphine to merit further investigation. Of the 3 doses tried, 60 mg/kg showed maximum protection with no mortality from the drug; this dose was used throughout the remainder of the work. The observed pattern of response to the injection of morphine was a preliminary excitation, lasting for 15 to 30 minutes, followed by a more prolonged depression, as described by Krueger, Eddy, and Sumwalt(7). Thus, the X-ray exposures were begun at the beginning of the depression period. The weight loss of the irradiated animals, though not accurately quantitated, was less in the morphine-treated groups than in the controls.

The results of the experiment designed to test the effect of morphine sulfate on the LD_{50} of whole-body X radiation are given in Fig. 1. Each point represents 40 mice except the 880 r control group and the 630 r and 670 r morphine-treated groups, which represent 20 mice. Results were calculated by the probit method. The figures indicate that if more experiments were carried out under conditions similar to those outlined above, in 95% of such experiments the LD_{50} of the untreated groups would fall in the interval between 595 r and 620 r. The interval for the morphine-treated groups is 819 r to 842 r. Since the confidence intervals for the 2 groups are far

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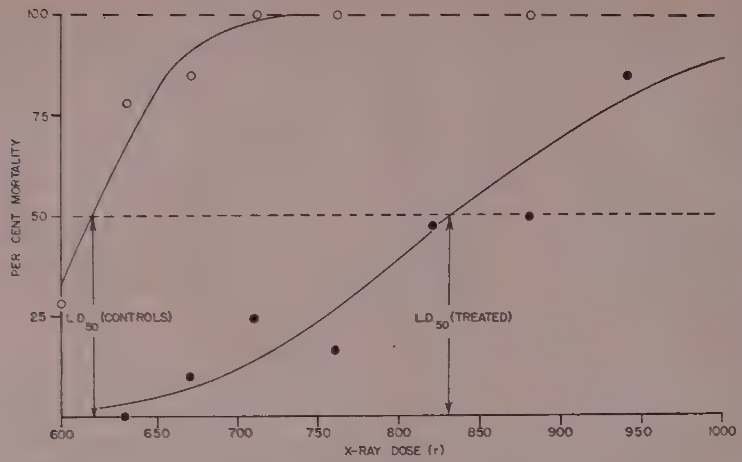

FIG. 1. Effect of morphine sulfate on LD_{50} of x radiation. ○ = control; ● = morphine-treated (60 mg/kg). Each point represents 20 to 40 mice.

from overlapping, it is clear without formal statistical treatment that the LD_{50}'s of the 2 groups differ significantly, at the level of 0.1% or less. Fig. 1 represents the probit fits to the observed mortalities, and indicates an increase in LD_{50} from 609 r in the controls to 830 r in the treated group.

Discussion. Morphine has long been known to affect many systems, such as the autonomic nervous system, central nervous system, and endocrines. However, only 2 of the actions of morphine seem to offer possible explanations of its effect in increasing the radioresistance of mice. The first of these is the hyperglycemic effect which morphine has been shown to have on various species(8), probably through an adrenal mechanism. Loiseleur and Velley(9) have shown that rats can be protected from the lethality of X rays by intraperitoneal injections of very large amounts of glucose 45 minutes before irradiation. Baclesse and Loiseleur(10) have demonstrated a reduction of cutaneous sensitivity of rabbits to X rays by intramuscular glucose injections. Both of these studies utilized exceedingly large doses of glucose near the lethal level; on the other hand, the rise of blood glucose after an injection of morphine is quite variable, and rarely exceeds 60 mg %. Hence, it seems unlikely that the hyperglycemic action of morphine can account for its protective ability.

The other effect of morphine which might serve to explain its protective action is its pronounced depression of the respiratory center. This depression of respiration results in a decrease of oxygen uptake by the blood, resulting in turn in decreased tissue oxygenation. This end effect is similar to that produced by placing animals in 5% oxygen(3), by producing methemoglobinemia(6), or by histotoxic anoxia(5). It is interesting that the curves of Fig. 1 have the same shape and the same difference in slope between graphs of control and treated mice as do the curves of Hollaender *et al.*(4) for the oxygen effect in *E. coli.* It seems likely that the mechanism of action of morphine in protecting mice against the lethal effect of radiation is a decrease of oxygen tension in some radiosensitive tissue or tissues. This parallels the conditions in *E. coli*(4) and other organisms(1,2).

The possibility remains that the protection afforded by morphine is on a purely physical basis, *e.g.*, through formation by morphine of a substrate for the action of activated products of water irradiation. This can be tested by injecting into mice an alkaloid structurally similar to morphine, but with different effects

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on the respiratory center. It is planned to test the effects of thebaine and of n-allylnormorphine in this manner, which should furnish further evidence for the role of the respiratory center in protection against radiation toxicity.

Summary. Morphine sulfate, injected intramuscularly into ZBC male mice 30 minutes before exposure to total-body X-radiation, raises the LD_{50} of the radiation from 609 r to 830 r. Possible mechanisms of this effect are discussed.

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Effect of Oral Terramycin Prior to Whole Body X-Radiation.* (19114)

GORDON E. GUSTAFSON AND SIMON KOLETSKY.

From the Institute of Pathology, Western Reserve University, School of Medicine.

Severe radiation injury is often complicated by bacteremia(1-3). The intestinal tract probably serves as the main portal of entry for organisms. This study was made to determine whether reduction or alteration in the intestinal flora by antibiotic therapy prior to radiation would be effective in lowering mortality.

Method. Two hundred white male rats of the C. F. Wistar strain having a mean weight of 195 g were separated randomly into 2 groups of 100 each. Animals were kept in cages having dimensions of 22 by 20 by 15 inches in groups not exceeding 10 rats per cage. Terramycin hydrochloride (10% aqueous solution) was administered orally, using a French No. 8 soft rubber catheter, by a modification of the technic described by Levin(4). The antibiotic was given in 5 doses of 100 mg each, at intervals of 10 to 14 hours, beginning 48 hours prior to radiation and ending 1 to 4 hours prior to radiation. No antibiotic was used after radiation. The rats were weighed 9, 3, and 1 day prior to x-radiation, daily for the first 6 days following radiation, and thereafter at 2 or 3 day intervals. The experiment was discontinued at the end of 30 days. X-radiation was carried out with a 250 KV, 15 MA unit at a distance of 44.3 cm using ½ mm copper and 1 mm aluminum filters. The dosage rate was approximately 60 r per minute. The total dose was 660 r.

Results. (1) Mortality. The 30 day mortality for the control group was 72% and for the treated group 48%. Analysis of the data by the method of Chi square gives a value of 4.08. This is statistically significant. (2) Survival. The mean survival time of the rats in the control group was 10.6 days as compared to 15 days for the treated group. The mean difference is 4.4 days and the mean standard error is 1.3 days. Statistically this difference is highly significant. Fig. 1 shows a plot of per cent mortality against days after x-radiation. The control rats began to die earlier than the treated rats and had a higher death rate up to the 12th day. From the 4th to the 12th day the mean death rate for the control group was 6.2% per day and for the treated group 2.6% per day. From the 12th to the 30th day the mean death rate for the control group was 0.9% per day and for the treated group 1.4% per day. However, when the death rate is calculated on the basis of the number of rats alive on the 12th day, the death rate for the control group is 2.3% per day and for the treated group 1.7% per day. (3) Morbidity. In general this paralleled the death rate. Morbidity was greater in the

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* This work performed under contract between the U. S. Atomic Energy Commission and Western Reserve University, Contract No. W31-109-eng-78. Terramycin furnished by courtesy of Chas. Pfizer and Co., Brooklyn, N. Y.

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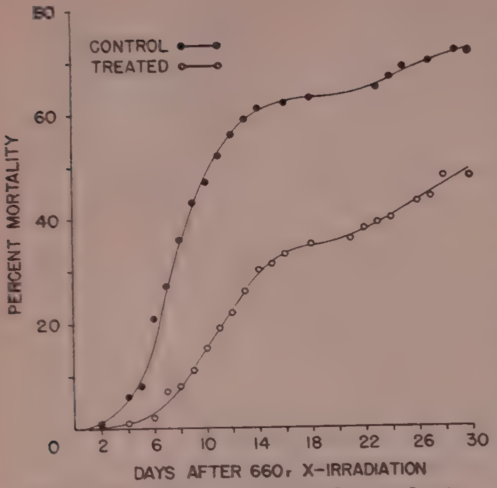

FIG. 1. Mortality in control and treated rats.

control group from the 4th to the 12th day and in the treated group from the 12th to the 30th day. Diarrhea was greater in the treated group than in the control group probably because of the large amount of terramycin which the former received. (4) Weights. The mean weights of the rats in the two groups were virtually identical except from the 5th to the 12th day post-radiation. During this period the terramycin rats gained weight faster than the controls. The maximum difference occurred from the 6th through the 8th day when the mean weight of the rats which received terramycin was 15 g greater than that of the controls. After the 12th day post-radiation there was no significant difference in the mean weights of the two groups.

Discussion. Koletsky and Christie(5) administered streptomycin and penicillin in combination parenterally to rats from 3 to 10 days prior to and for 3 weeks following injection of rats with lethal doses of P^{32}. In another group of animals the use of antibiotics began immediately after the P^{32} was injected and continued for 3 weeks. The treatment was effective in increasing the survival time and decreasing the mortality from internal radiation. Miller and Hammond(6) and Hammond and Miller(7) used several antibiotics effectively in irradiated mice, *i.e.*, streptomycin, streptomycin combined with penicillin, chloromycetin, terramycin and aureomycin. The last three were employed orally and also by parenteral route. Therapy was usually started on the first day post-radiation and continued through the 19th or 24th day, although in two experiments chloromycetin and aureomycin respectively were given orally from the 4th through the 24th day. The best results were apparently obtained with streptomycin alone. Furth, Coulter, and Howland(8) gave antibiotics from the time of x-radiation through the 28th day. They found no significant difference in the 28 day mortality with aureomycin but stated that terramycin appeared to be effective in reducing mortality. The most obvious result of the antibiotics was to decrease the incidence of post-radiation diarrhea.

In this study prophylaxis with oral terramycin for only 48 hours prior to x-radiation proved beneficial. By altering the intestinal flora, the antibiotic presumably reduced the incidence of bacteremia following radiation. Terramycin may be even more effective when used for 72 rather than 48 hours before radiation. In an experiment employing pre-treatment for 72 hours, the 30 day mortality for treated rats given 660 r of whole body x-radiation was 32% as compared to 86% for the control animals(9).

Summary. Terramycin has been shown to be effective in reducing the mortality in rats when administered for a period of only 48 hours prior to 660 r of whole body x-radiation.

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Antigenic Similarity of Some Trained Resistant Strains of Viridans Streptococci to *Streptococcus fecalis*.* (19115)

MAXINE FELTZ AND WILLIAM E. CLAPPER.

From the Department of Bacteriology, University of Colorado Medical School, Denver.

It has been shown in this laboratory(1) that some strains of viridans streptococci which were trained to resistance to sulfathiazole, penicillin, and aureomycin acquired many biochemical properties characteristic of enterococci. Trained streptomycin-resistant strains varied in their acquisition of these properties. Since the enterococci possess a common antigen, it seemed of interest to determine whether these resistant strains were antigenically related to a known strain of *Streptococcus fecalis*. This was found to be the case.

Experimental. Seven strains of viridans streptococci were used as the parent organisms. Four were obtained from saliva, one from urine, one from blood, and one from the pericardium of 7 different individuals. The resistant strains were prepared by repeated transfers in broth containing the various agents(1). The penicillin and aureomycin-resistant strains were retested and found to be resistant to at least 100 units per ml. The streptomycin-resistant strains were transferred once in broth containing 100 units per ml of streptomycin to insure a high resistance, since previous experience had indicated that this type of resistance may be lost after successive transfers in the absence of the antibiotic. All the parent strains were susceptible to 1 unit or less of penicillin and aureomycin, and to 5 units or less of streptomycin. None of these strains were able to grow in 0.1% methylene blue milk and on 40% bile agar, while all of the resistant strains were able to grow.

Preparation of materials for precipitin tests. The immune serum was prepared by injecting rabbits with formalin treated *Streptococcus fecalis* (ATCC No. 9790) cells. Lancefield's (2) and Fuller's(3) methods of extracting the antigen from the cells were tried with negative results. However, when the extraction was carried out after treating the cells with the enzyme preparation of *Streptomyces albus*, according to the method of Maxted(4), positive results were obtained. The precipitin tests were carried out by drawing undiluted antiserum into capillary tubes, followed by the extract containing varying dilutions of the antigen. The tubes were incubated one-half hour at 37°C, then placed in the refrigerator overnight, and the presence or absence of precipitate observed with the aid of a widefield microscope having a magnification of 6.3X. The various strains were grown for 18-24 hours in 10 ml of Bacto-Difco brain heart infusion broth, and adjusted to a turbidity of 200 on the Klett-Summerson colorimeter before further treatment. Each organism was cultured, harvested, and the antigen extracted from 3 to 6 different times.

Results and discussion. It was not possible in every case to obtain a positive precipitin reaction with our antiserum and the enterococcus used as the immunizing antigen. However, of 37 such tests, 26 were positive, and all 3 tests performed with another known *Streptococcus fecalis* were positive. Readings were made only when controls were positive. Table I shows that the sulfathiazole susceptible viridans strains did not give a precipitin reaction with antiserum prepared against *S. fecalis* No. 9790. The 2 strains which gave biochemical tests of viridans streptococci but were more resistant to sulfathiazole did react. When the parent strains were trained to resistance to sulfathiazole, aureomycin, and penicillin they acquired the ability to react with *S. fecalis* antiserum as well as other properties of enterococci. One of the 5 streptomycin-resistant strains did not react with the

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TABLE I. Precipitin Reactions of Parent and Resistant Strains with *S. fecalis* Antiserum.

	Parent strains		Sulfathiazole resis. strains		Penicillin resis. strains		Aureomycin resis. strains		Streptomycin resis. strains	
Culture number	Inhib. con. sulfa	% tests pos.	Inhib. con. sulfa	% tests pos.	Inhib. con. sulfa	% tests pos.	Inhib. con. sulfa	% tests pos.	Inhib. con. sulfa	% tests pos.
1	.1*	0†	30	66	30	100	30	66	30	100
4	.1	0	30	50	30	100	30	66	>30	66
6	.1	0	30	40	30	100	>30	66	30	66
17	10	33	30	80	30	100	>30	66	10	0
27	30	66	>30	100			>30	66		
34	1	0	>30	60						
57	1	0	30	50	30	66	>30	66	>30	33

* mg/ml of sulfathiazole required to inhibit growth.

† 3 to 6 tests performed on each organism.

antiserum. It was somewhat less resistant to sulfathiazole than were the other strains. We have reported that streptomycin-resistant strains, unless trained to a very high resistance, may not become as highly resistant to sulfathiazole as do other antibiotic-resistant trained strains from the same parents(1). Other biochemical properties appear to change more slowly, also, in this group of trained organisms.

Yegian, Budd, and Middlebrook(5) could demonstrate no change in antigenicity in a sulfonamide-resistant strain of *Mycobacterium ranae*. Gezon and coworkers(6-9) found some changes to occur in B-hemolytic streptococci when they were made resistant to penicillin, but little or no change when made resistant to aureomycin, bacitracin, and streptomycin.

We have now shown that viridans streptococci may be trained to resistance to sulfathiazole or to resistance to several antibiotics, and if they become sulfathiazole resistant at the same time, these strains become, in all the properties tested, exactly like enterococci. These properties include the ability to reduce methylene blue milk, to grow in 40% bile agar, and in broth containing high concentrations of sulfathiazole or of penicillin(1), to produce CO_2 from arginine and tyrosine(10), and to produce a positive precipitin reaction with *S. fecalis* antiserum. Sulfathiazole-sensitive viridans strains do not have these properties. There can be little doubt that the various agents used to select the resistant strains induced a change in the original organisms, since the resistant strains grow much more rapidly than the parent strains. If a few had been present in the original cultures, these would have grown out by transferring in broth in the absence of the inhibitors. Such a change was not found in any of our strains upon successive transfer in broth. Furthermore, planting a few resistant organisms in a culture of the parent strain resulted in a resistant culture after one transfer. It would certainly seem that several strains of viridans streptococci have been induced to change to enterococci.

Summary. Antigens similar to those found in a strain of *S. fecalis* were found in all but one of the sulfathiazole, aureomycin, penicillin, and streptomycin-trained resistant strains of viridans streptococci. The antigens were not present in 5 of the parent strains with low sulfathiazole resistance but were present in 2 with higher resistance.

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Tritium-Induced Effects in *Paramecium Aurelia* (19116)

E. L. Powers and Deborah Shefner. (Introduced by A. M. Brues.)
From the Division of Biological and Medical Research, Argonne National Laboratory, Chicago, Ill.

Measurements of genetic effects of certain metabolized radioactive isotopes are of practical interest in the general evaluation of their toxicity and of theoretical interest in that they may lead to clues of the nature of genetic mutations. This report records observations on the genetic effects of tritium (T or H^3), one of these isotopes, in *Paramecium aurelia*.

Methods. The preparations were made by mixing 1.8 ml culture fluid(1) and 0.2 ml water containing suitable concentrations of tritium oxide. Distilled water was added to the fluid in which the controls were grown. These solutions were inoculated with a small drop of a suspension of *Aerobacter aerogenes* which was allowed to grow at room temperature about 14 hours. Then about 0.8 ml of the bacterial suspension was transferred to a deep depression slide, a single paramecium was introduced, and a cover slip was sealed with petroleum jelly over the depression to prevent evaporation of the radioactive water. The animals were allowed to grow in these preparations for about 48 hours. The final activities of each of the solutions and the incubation periods are given in Table I. In any one experiment, the animals were sister cells derived from one recently out of autogamy and, therefore, presumably homozygous(2). All animals were of Variety 4, mating type VIII, containing kappa(2). After the exposure period, the organisms were washed 5 times in inactive culture solution by serial transfers with micropipettes, diluting the original activity by a factor of about one million. From the group of washed animals a number of cells were isolated into separate depressions (Table I). The isolates grew until they had exhausted the food (3-5 days) in approximately 1 ml culture fluid, at which time the members of a clone generally entered autogamy as a unit. From each autogamous culture 36 animals were isolated and, after an incubation period of 4 days, observed for survival. While it is difficult at times to distinguish between surviving and nonsurviving clones, it was found satisfactory to classify as nonsurviving any isolate which, during the period of 4 days, did not give rise to a culture of at least 60-70 animals with few or no abnormal cells. In Table I the statement of effect (% death) is an average of the % death after autogamy induced in all the individual isolations from each treatment. Thus, the first line of Table I indicates 16 isolations from the treatment; at autogamy 36 isolations were made from each of these 16 (576 in all); and 11 of the 576 did not survive (1.9%). The activity of the stock solution of T was assayed by reducing the water to H_2 and measuring the ionization within a chamber caused by a known volume of the H_2 gas* (error not larger than 5%). The radiation dosage delivered by long-lived electron emitters homogeneously distributed in volumes that are large with respect to the range of the particles may be calculated(3)† according to rep/day = 55 E C, in which E is the average energy of the β particles in electron volts, and C is the activity in curies. In the case of T, with an average energy of 5.69 kev(4), the dosage is 0.313 rep/day/μc/ml.

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† The formula given is actually 61 E C; however, the constant 61 contains the number 83 as the energy in ergs dissipated in a gram of tissue corresponding to one roentgen unit gamma radiation in air. It is now generally agreed that 93 ergs/gm is more nearly accurate, and 55 E C is offered as a closer approximation of the equivalent dosage. See Evans, R. D., *Nucleonics*, 1947, 1, 2, 32.

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TABLE I. Death After Autogamy Following Growth in Tritium-Containing Solutions.

Exp. No.	T conc., mc/ml	Exposure time, hr	Dosage, rep	No. exp. isolations	Avg % death after autogamy Treated	Control
2	1.03	47	630	16	1.9	.8
1	1.03	48	645	23	1.7	.6
3	5.15	44	2955	27	19.6	1.2
3	10.30	44	5900	27	28.8	1.2
1	10.30	48	6445	15	37.3	.6
4	13.90	48	8700	23	65.2	.7
3	15.45	44	8860	23	62.5	1.2
3	20.60	44	11810	25	64.8	1.2
4	32.45	48	20300	26	96.4	.7
4	46.35	48	29000	24	93.9	.7
2	103	47	63100	18	98.1	.8
1	103	48	64450	10	73.3	.6

The dosages shown in Table I for the various treatments were calculated on this basis.

Results. In regard to number of divisions during treatment (3-4 fissions/day), vegetative death of cells (0%), and the growth behavior after treatment until autogamy, the treated animals did not differ from the control animals with the exception of the experiment reported in the last line of Table I. There was an unexplainable preautogamous depression of growth rate in the treated animals which might be related in some way to the atypically low death after autogamy observed in these animals.

With increasing activity of T in the culture solution, an increasing degree of death after autogamy was observed. The response seems to be linear up to the region of 15,000 rep (Fig. 1). This type of response has been observed for other isotopes and X rays(5,6). In view of results in Variety 1 of this species (7) which demonstrate a sigmoid type of response, the question as to the precise nature of the curve is left open. However, for purposes of comparing it with the response induced by other isotopes, a straight line function for the first 8 points was assumed, and the best straight line was calculated. The line is $y = 6.34\,x - 1.1$; y being death after autogamy in % and x being radiation dosage in thousands of rep. The standard deviation of the slope (σ_b) is 0.69.

The $Sr^{89,90}Y^{90}$ experiments with which the T response is compared are from an earlier study(5). It had been established that equivalent absorbed energies from $Sr^{89,90}Y^{90}$ and X rays(6) produce approximately equivalent results, and in order to demonstrate that the response of the animals at the time of the T experiments was similar to that observed in the Sr experiments, several X-ray control studies were made. Previous experience indicated that 10,000 r X radiation induced 76% death after autogamy, measured by our criteria of death. At the time of the T experiments, two separate exposures to 10,000 r (200 kvp X rays through 0.25-mm Cu and 3-mm Bakelite at 400 r/min) involving 53 isolated treated animals and 1900 isolations after autogamy yielded an average of 71% death after autogamy. This result is well within the error of the X-ray line, and it was concluded that a comparison of these experiments with the earlier ones is permissible.

The Sr response, also assumed to be linear,

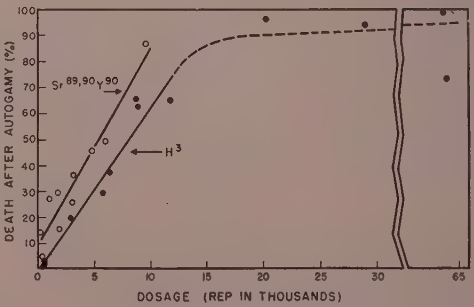

FIG. 1. Comparison of effect of $Sr^{89,90}Y^{90}$ and of H^3 on death after autogamy.

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which is shown in Fig. 1 is described by $y = 7.70\,x + 8.5$; with a σ_b of 0.86. The high value of the y-axis intercept is consistent with the higher control deaths observed at that time. Comparison of the slopes of these two responses reveals no difference between them, the t value being 1.24 with 14 degrees of freedom indicating $0.20 < P < 0.30$.

Discussion. It has been shown that the macronuclei of exautogamous cells are derived from the micronuclei of preautogamous cells under the conditions of these experiments, and that the process of autogamy ensures homozygosity and expression of the genetic constitution of the micronuclei(2), but it is not as certain that all death following autogamy subsequent to treatment by a mutagenic agent is due to disturbances in the nuclear genetic system. The nature of this death has been examined in Variety 1 of *P. aurelia* by Kimball(8) who shows that while the factors causing low vigor and death for the most part are inherited as if the micronuclei have been damaged in some way, there were certain departures from expectation that are unexplainable at the present time. These exceptions were observed in one experiment and involved a small percentage of the total number of crosses analyzed. While these exceptions must be taken into consideration and while all death after autogamy observed in experiments reported here is not necessarily due to lethal micronuclear aberrations expressing themselves in the usual manner, Kimball's analysis permits us to regard a great portion of the death after autogamy as being due to lethal changes of some kind in the micronuclei of exposed animals. It is not unreasonable to believe that two mutagenic agents of the same type induce proportional amounts of regular genetic damage even though the actual extent cannot be measured accurately.

On the assumption that the biological effect induced by T and Sr-Y is, for the most part, an expression of genetic damage, we can examine the significance of the result. There are two reasons to suspect beforehand that the effects of T may be different in degree from those of certain other radioactive isotopes, the biological system receiving the same total radiation dosage from all.

First, there is dependence of genetic effect on the kind of radio-element. It has been shown that P^{32} induces death after autogamy much more efficiently than $Sr^{89,90}Y^{90}$(5), that part of the increased efficiency is ascribable to accumulation of P^{32} in the cell and a corresponding increase in dosage to the cell, and that the accumulation phenomenon probably does not account for all the excess effect of this isotope(9). The likely interpretation of such a result is that effects are being induced as a result of the transmutation of P^{32} to S^{32} and the associated recoil occurring at the time of expulsion of the electron from the nucleus of the atom. The same kind of evidence has been reported for bacteria(10) and bacteriophage(11). This effect must be in excess of that induced by β particles, and it depends on the number of radioactive atoms present in strategic spots and the disintegration constant of the isotope. In the case of T, the relative mass difference between it and H, which it is replacing, is very great. This difference could result in much lower specific reaction rates for T and compounds containing it. In consequence there would be differential selection by enzymatic systems of non-tritiated compounds and a resulting frequency of T in H positions below the expectation indicated by the T/H ratio offered to the organism for use (this effect is known for deuterium). Since specific activities (T/H) have not been measured in these experiments, a critically proved statement cannot be made; the apparent lack of a transmutation effect is expected if it is small and there is biochemical selection against T in the organism. Therefore, it appears that if transmutation of the incorporated T contributes to the genetic damage being measured, it is so small that it is unrecognizable

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even after growth of the cell through 7 cell generations in concentrations of T up to 100 mc/ml.

The second reason for uncertainty in predicting the response of the organisms to exposure to T stems from ignorance of the role of specific ionization in the induction of the effect. An electron with an average energy of 5.69 kev (T) produces on the average 184 ion pairs per micron of track (12p.24), while one of 700 kev ($Sr^{89,90}Y^{90}$) produces about 12 ion pairs per micron of track(13). This difference obtains in these experiments. Zirkle(14) has examined the literature in some detail and agrees that with an increase in specific ionization, total radiation dosage remaining the same, the observed decrease in lethal effect of ionizing radiations on small systems such as vegetative bacteria and viruses is real. In the case of induction of lethal mutations, however, the situation is not as clear. Lea(12) states that the evidence to date warrants the conclusion that the differences in mutation rate (X chromosome recessive lethals) noted by several investigators following treatment of *Drosophila* with agents producing varying degrees of specific ionization in tissue are significant, and are indicative of an inverse dependence of mutation induction on the number of ion pairs per unit length of track. However, because of the difficulty of estimating dosages from α particles, soft radiation, and neutrons to the germinal tissue of *Drosophila*, the evidence is not as convincing as it is in the case of bacteria and viruses.

If the small difference in slope of the two response lines described here is considered real, we note that the ratio (slope T/slope Sr) of the rates is 0.82. This ratio is entirely consistent with Lea's summary (p. 153) of the relationship of mutation yield and increasing ion density of radiation. Indeed, it is very near the value of 0.78 reported by Wilhelmy *et al.*(15) for the relative effectiveness of X rays of 6.2 kv effective compared with those of shorter wavelengths. This correspondence may be significant since the ion density produced by electrons from T (average of 184 ions pairs/μ) is close to that expected from X rays of this low energy. It should be noted, however, that these authors were uncertain of their dosage measurements, and our numbers do not indicate a real difference in response. Thus, while there may be some significance in the coincidence, the similarity of the results may be fortuitous.

Because no information on the distribution of T within the cell is available, a dosimetry question which may have bearing on the interpretation must be considered. If the micronuclei contain no radioactivity they can be considered spherical "holes" in the radiation field, receiving a dose from the surrounding cytoplasm.‡ In the case of energetic electrons such as those arising from the Sr, Y isotopes, the 55 E C formula is usable for calculating the dose delivered to the "hole." But when the energy (and, therefore, the length of track) becomes small relative to the radius of the "hole," the calculation is uncertain. In the condensed interphase stage the micronuclei are spheres of 0.75 μ radius, and the length of path of a 6-kev electron (the mean energy of the tritium beta particle) in water is cited as 1 μ (12,p.24). There is, then, conceivably delivered to the center of the micronucleus, if it contains no tritium, a dose smaller than that indicated by the calculation. Until there is available a description of the absorption in water of tritium β particles, calculation of the precise dosage to the micronucleus in these circumstances is impossible. However, it is highly unlikely that the micronuclei contain no tritium, since they are duplicated 6 or 7 times during the exposure period within

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‡ This and similar problems of radiation dosage from radioactive solutions are discussed by Richards, P. I., and Rubin, B. A., *Nucleonics,* 1950, v6, 42 and by Rossi, H. H. and Ellis, H. Jr., *Nucleonics,* 1950, v7, 18, 19.

cytoplasm which, according to our observations on the rate of water output by the contractile vacuoles, is nearly equilibrated with the aqueous radioactive environment within 30 minutes after the beginning of exposure. Nevertheless, a definite answer to this dosage question must await solution of the difficult problems of microdistribution of T within the cell and the absorption of very soft β particles in water.

Summary and conclusions. Effects induced by metabolized radioactive hydrogen (tritium or T) and presumed to be genetic in nature have been measured in *Paramecium aurelia.* Organisms were allowed to divide 6 to 8 times during 2 days in culture fluid containing T in concentrations varying from 1 to 100 mc per ml. With increasing radioactivity of the medium, increasing death after autogamy was observed, but no preautogamous effects were noted. The response observed after exposure to T appears to be slightly lower than that induced by emitters of more energetic β particles at similar dosage levels. The conclusions are that no part of the effect can be ascribed to transmutation of T, the emitted electrons alone apparently inducing the biological changes; and that the difference in efficiency between T and $Sr^{89,90}Y^{90}$ in inducing the effects observed is consistent with the observations of others concerning the dependence of the mutation constant upon rate of energy loss by densely ionizing particles.

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Electron Micrographs of Newcastle Disease Virus Preparations from Chick Embryos Following Infection by Virus Propagated in the Short-tailed Shrew (*Blarina brevicauda*). (19117)

REGINALD L. REAGAN AND A. L. BRUECKNER.

From the Live Stock Sanitary Service Laboratory, Md. State Board of Agriculture, University of Maryland, College Park.

The short-tailed shrew is one of the smallest of the mammals and has an extremely high rate of metabolism. Electron microscope studies were conducted to determine the morphology of Newcastle disease virus (NDV) recovered from this sensitive mammal following infection by intracerebral inoculation and intranasal instillation. In previous experiments tailed virus forms were demonstrated from purified egg-adapted virus material by Bang(1,2) and Cunha *et al.*(3). Tail-like forms were also demonstrated in hamster-adapted, mouse-adapted, and bat propagated NDV after culture in 10-day embryonated eggs by Reagan and his coworkers(4,5)

Procedure. Three-hundredth ml of allantoic fluid from 10-day embryonated White Leghorn chicken eggs infected with the 25th egg passage of California strain (No. 11,914) was inoculated into the right frontal lobe of each of three *Blarina brevicauda* short-tailed shrews. Three-hundredth ml of the same material was instilled intranasally in each of 3 additional shrews. Typical Newcastle disease symptoms developed within 24 to 30 hours in the shrews infected intracerebrally and within 5 to 6 days in the shrews infected nasally. The symptoms from both series were irritability, malaise, rhythmical contraction of muscles starting in the limbs and spreading over the entire body, and

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FIG. 1 (left). Newcastle disease virus from the brain of an infected short-tailed shrew (intranasal instillation) *Blarina brevicauda* (1 s.c.), after one subculture in embryonated eggs, shadowed with chromium at tangent 1/6. × 32,000.

FIG. 2 (right). Egg-adapted Newcastle disease virus in allantoic fluid of embryonated chicken eggs, shadowed with chromium at tangent 1/6. × 32,000.

paralysis of the fore and hind legs followed by complete prostration. There was no evidence of excessive salivation from pharyngeal paralysis which is characteristic in other animals infected with the hamster-adapted NDV(6).

When nervous symptoms developed in each shrew the brain was removed aseptically, ground in a mortar with alundum, and diluted to a 10% suspension with physiological saline. This virus-bearing material from each series was inoculated separately into the allantoic sac of 10-day embryonated chicken eggs. All embryos died within 72 hours. A pool of virus-bearing allantoic fluid was prepared for each series. The presence of virus in this material was verified to be that of Newcastle disease by neutralization tests using embryonated chicken eggs as the test host. Each pool of allantoic fluid was centrifuged in a horizontal centrifuge for 5 minutes at 2,000 rpm. The sediments were discarded and the supernatants were recentrifuged for 2 hours at 44,040 rpm in a Spinco ultracentrifuge. There was less than ½ degree change in the temperature of the refrigerated outer jacket. The sediment from each series was resuspended in 3% saline for 10 minutes and then spun lightly for ½ minute at 1,000 rpm in a horizontal centrifuge. The supernatant from each series was placed separately on parlodion-prepared screens (which had been prepared 24 hours previously), shadowed with chromium(7-9) at arc tangent 1/6 and examined under the RCA EMU electron microscope.

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Numerous fields chosen for electron examination showed many virus particles with tail structure. Fig. 1 shows broad tail forms from the intranasal shrew passage after virus cultivation in embryonated eggs. Virus particles from the intracerebral series appeared similar to those from the intranasal series. Fig. 2 shows an electron micrograph of the egg-adapted California strain carried only in embryonated eggs.

Summary. (1) NDV from short-tailed shrews infected by intracerebral inoculation and intranasal instillation was 100% pathogenic for embryonated eggs in the first embryo passage. Shrews infected by these two methods of exposure showed typical Newcastle disease symptoms. The virus in the allantoic fluid of eggs inoculated with the shrew brain material from both intracerebral and intranasal series was neutralized by positive Newcastle disease serum but was not affected by normal chicken serum. (2) Electron micrographs of Newcastle disease virus preparations from chick embryos following infection by the virus propagated in the short-tailed shrew showed a predominance of tailed forms. (3) Intracerebral and intranasal serial passages of NDV in the short-tailed shrew are being continued at the present time and will be reported in a later paper.

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Resistance in Leukemic Cells to a Guanine Analog, 8-Azaguanine. (19118)

L. W. LAW. (Introduced by H. B. Andervont.)
(With the technical assistance of Peter J. Boyle.)
*From the National Cancer Institute, Bethesda, Md.**

The development of resistance in mouse leukemic cells, lymphocytic in type, to folic acid antagonists has been reported(1,2). The resistant variants, in certain cases, proved to be partially dependent upon folic acid antagonists for optimal growth(3,4). Experimental evidence favors the assumption that specific changes in the hereditary mechanism of the cell have occurred, independent of the action of antagonists. The transformations produced were found to be stable, irreversible and heritable(4) and occurred generally in large populations of leukemic cells(5). The triazolopyrimidine analog of guanine, 5-amino-7-hydroxy-1H-v-triazolo (d) pyrimidine (8-azaguanine) has been shown to be moderately inhibitory to certain bacteria(6) and to *Tetrahymena*(7,8) and a definite inhibitory action toward certain experimental tumors has been observed(9) and confirmed (10-12). It is quite clear, however, that this metabolic inhibitor is entirely inactive against other tumors in mice and rats(10,11,13,14).

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* National Institutes of Health, Public Health Service, Federal Security Agency.

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TABLE I. Influence of 8-Azaguanine on Survival Time of Mice Bearing Transplants of Sensitive (Control) Leukemia L 1210.*

Exp.	Treatment	Strain of mice	No. mice	Dosage (mg/kg) Daily	Dosage (mg/kg) Total	Mean survival time (range) in days	% increase in survival time
1	Control	dba	10	0		10.1 (9-12)	
	8-Azaguanine		10	75	900	17.3 (16-19)	71.3
2	Control	dba	10	0		10.1 (9-12)	
	8-Azaguanine		10	75	900	17.1 (15-19)	69.4
3	Control	dba	10	0		8.8 (8-9)	
	8-Azaguanine		10	75	750	14.1 (13-16)	60.2
4	Control	BDF$_1$	8	0		10.6 (8-14)	
	8-Azaguanine		8	75	975	17.1 (11-25)	61.3

* Inoculations of a standard dose of leukemic cells done intraperitoneally. Injections of 8-azaguanine begun 24 hr post-inoculation. BDF$_1$ mice = ♀ C$_{57}$ black × ♂ dba.

In certain acute lymphocytic leukemias of the mouse(12), particularly the transplantable leukemia, L 1210, a definite, regular and reproducible inhibition of growth results from parenteral administration of the guanine analog at near maximum tolerable doses (Table 1). It was of interest, therefore, to determine whether resistance to this anti-leukemic compound similar to resistance to anti-folics could be developed.

This report offers evidence for the development of resistance to 8-azaguanine and partial dependence for growth on this guanine analog in leukemia L 1210 and presents some of the pertinent characteristics of these resistant and dependent leukemic cells.

Transplant generations 100 to 124 of the sensitive subline of leukemia L 1210(15) have been used as control material for the present study. Beginning with the 100th transfer, a standard dose of leukemic cells (8 x 10^5 cells) in Lockes' solution was inoculated subcutaneously in the right axillary region, into dba strain mice which subsequently received daily injections of 75 mg/kg of 8-azaguanine. The guanine analog was dissolved in 0.01 N NaOH, and was injected subcutaneously on the left side, 0.05 cc per gram body weight. Injections were begun 24 hours following transfer of leukemic cells and were continued until the next transfer. As can be seen from Table II, during the first 7 serial transfers, subcutaneous growths were obtained for further transfer at from 13 to 18 days. At the 8th transfer, however, palpable growths were obtained at the 4th post-inoculation day and at 9 days the mean weight of the lymphomatous mass was 573.9 mg. Subsequently through the next 16 transfers sizable tumor growths were obtained in mice receiving a total dose of 600 mg/kg of 8-azaguanine, indicating the development of resistance. In contrast, identical transfer generations of the control subline, carried in untreated mice, remained sensitive to 8-azaguanine, showing nearly complete inhibition of growth of lymphomatous tissue at 9 days (Table II).

Partial dependence for growth of these resistant leukemic cells on the guanine analog is shown at transfers 18, 19, 21 and 23 in Table II. Significantly greater mean tumor weights were obtained in mice supplied with a total dose of 600 mg/kg of 8-azaguanine in contrast to mean weights of dba strain mice growing the resistant cells but deprived of the analog. Differences in survival time of mice growing the resistant line of leukemia L 1210, and supplied with or deprived of analog, also reflect a degree of dependence (Table III).

The possibility was considered that resistance to 8-azaguanine might extend to other agents exerting specific anti-leukemic effects. It can be seen by reference to Table II, transfers 19 and 21, that resistance to 8-azaguanine

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TABLE II. Response of 8-Azaguanine-Resistant and Sensitive (Control) Lines of Leukemia L 1210 to 8-Azaguanine and Other Anti-Leukemic Compounds.

Transfer generations	Strain of mice	No. mice	Time transplanted, days	Compound	Dosage (mg/kg) Daily	Dosage (mg/kg) Total	Tumor wt (mg) at 9 days	
				8-Azaguanine-resistant				
G−1 to 7	dba	3 each	13–18	8-Azaguanine	75	825–1500	0	
8	,,	5	9	,,	75	600	573.9	
9	,,	10	,,	,,	,,	,,	173.6	
10	,,	5	,,	,,	,,	,,	623.3	
11	,,	10	,,	,,	,,	,,	287.3	
12	,,	10	,,	,,	,,	,,	164.4	
G–13 to 15	,,	3 each	,,	,,	,,	,,	Carried only	
16	,,	5	,,	,,	,,	,,	725	
17	,,	9	,,	,,	,,	,,	632.8	
18	,,	5	,,	0	0		545.6 ± 48.6	2.7*
		5		8-Azaguanine	75	600	204.6 ± 26.8	
19	BDF$_1$	10	,,	,,	,,	,,	382.1 ± 46.8	1.4
		10		0	0		274.5 ± 32.1	
		10		A-Methopterin†	3	12	8.8	
20	dba	5	,,	8-Azaguanine	75	600	735	
21	,,	10	,,	,,	,,	,,	462.6 ± 39.4	2.3
		10		0	0		205.2 ± 21.2	
		10		TEM	.5	2	129.4	
		10		A-Methopterin	3	12	5	
22	,,	5	,,	8-Azaguanine	75	600	700	
23	,,	10	,,	,,	,,	,,	776.9 ± 27.5	2.4
		10		0	0		318.1 ± 40.2	
				8-Azaguanine-sensitive (control)				
8	dba	10	,,	8-Azaguanine	75	600	43.5	
		10		0	0		682.8	
16	,,	10	,,	8-Azaguanine	75	600	26.7	
		10		0	0		1190	
19	BDF$_1$	10	,,	8-Azaguanine	75	600	10	
		10		0	0		528.3	

* Dependency ratio: $\frac{\text{Mean tumor wt in mice given analog}}{\text{Mean tumor wt in antagonist-free mice}}$.

† A-methopterin and TEM given intraperitoneally every other day beginning at 24 hr post-inoculation.

TABLE III. Comparison in Response of 8-Azaguanine-Resistant and Sensitive Leukemic Lines to 8-Azaguanine.*

	Line of leukemia	Dosage (mg/kg) Daily	Dosage (mg/kg) Total	Survival time, days	Peripheral blood counts Hb	wbc	agran.	gran.	blasts	Organ infiltrations Lymph nodes	Spleen	Liver
1	8-Azaguanine resistant	75	675	9.8 ± .16†	10.3	127150	(16%) 20344	(43%) 54675	(41%) 52131	3+	3+	3+
2	sensitive	75	675	See table I	15	18080	(53%) 9582	(44.7%) 8082	(2.3%) 416	—	—	—
	,,	None			9.2	91610	(30%) 27483	(48.5%) 44430	(22.5%) 19697	3+	3+	2+

* Inoculations of a standard dose of leukemic cells (8×10^5) done intraperitoneally. All determinations are at 9 days post-inoculation. Blood and histologic studies represent means of 5 mice each and survival times were determined on 20 mice in each group.

† In contrast, dba mice growing 8-azaguanine-resistant cells and deprived of analog had a mean survival time of 11.6 ± .31 days.

was not extended to 4-amino-N^{10}-methyl PGA (A-methopterin) nor to 2,4,6-triethylene-imino-s-triazine (TEM), these resistant cells being sensitive to the two compounds.

The morphology and biologic behavior of the 8-azaguanine-resistant subline grown in dba strain mice receiving 8-azaguanine resembles the sensitive control line grown in antagonist-free mice. At 9 days, in addition to growth of lymphomatous tissue subcutaneously, there results a florid leukemia with severe infiltration into lymph nodes, spleen and liver, leukocytosis with escape of lymphoblasts into the blood and a severe secondary anemia. The mean survival time of mice bearing the resistant and dependent cells and given 8-azaguanine is strikingly similar to the control line. Table III gives the comparative blood picture of dba mice growing the sensitive and resistant leukemic cells, the histologic pattern of infiltration and a comparison of the mean survival times of these groups.

It has been assumed by Kidder and coworkers(9) that neoplasms, unlike normal tissues, require guanine for growth and that 8-azaguanine acts as a competitive inhibitor of this changed metabolism of guanine by cancerous tissues. Results have been equivocal, however, relating to the question as to whether or not free guanine is metabolized by cancerous tissues in contradistinction to normal tissues(16-18). It is hoped that the variant leukemic cells described here, which are resistant to and partially dependent on 8-azaguanine for growth, will offer more suitable material for studies on the role of guanine in cancer.

Summary. (1) The development of resistance in a transplantable acute lymphocytic leukemia of the mouse to a guanine analog, 8-azaguanine, is reported. (2) Optimal growth of the resistant cells is attained in mice receiving near maximum tolerable doses of 8-azaguanine, indicating partial dependence of the variant line on the analog used in developing resistance. (3) The sensitive (control) line of leukemia has shown nearly complete inhibition of tumor growth at comparable transfer generations. (4) A-methopterin and TEM inhibit growth of the resistant line. (5) The biologic behavior of resistant leukemic cells, grown in mice receiving 8-azaguanine, strikingly resembles sensitive cells grown in antagonist-free mice: A florid leukemia develops with leukocytosis and escape of lymphoblasts into the blood, severe infiltration into lymph nodes, spleen and liver, resulting in death at approximately the same time as controls.

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Protein Metabolism of Amphibian Embryo. III. Incorporation of Methionine into Protein of Gastrulae.* (19119)

RICHARD M. EAKIN, PHYLLIS B. KUTSKY, AND WILLIAM E. BERG.

From the Department of Zoology, University of California, Berkeley.

An increasing number of studies is being made of regional differences in the physiology of the amphibian gastrula which may eventually elucidate the general problem of embryonic induction and, specifically, the mechanism whereby dorsal mesoderm (organizer of Spemann) evokes the differentiation of neural tissue from ectoderm. Friedberg and Eakin(1) showed that C^{14} of glycine is more rapidly incorporated into protein or intermediary compounds by the dorsal halves of gastrulae and neurulae than by the ventral

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* Supported by a research grant from the National Institutes of Health, U. S. Public Health Service.

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TABLE I. Incorporation of Methionine S^{35} by Dorsal and Ventral Half-Gastrulae.

				Dosage		Radioactivity of protein methionine			
			No. half			cts/min/μ mol sulfur		% activity	
Series	Exp.		embryos	mg methionine	μc S^{35}	D*	V*	D	V
I	*Rana pipiens*	1	200	.64	3.2	64	51	56	44
		2	150	.64	2.8	56	41	58	42
		3a	140	.65	2.5	92	80	54	46
		3b	140	.65	2.5	117	70	63	37
		4a	150	.70	2.4	78	69	53	47
		4b	150	.70	2.4	95	72	57	43
II	*Hyla regilla*	5	200	.70	2.9	68	63	52	48
		6	200	.61	2.1	65	62	51	49
		7	200	.70	2.0	77	60	56	44

* D = dorsal; V = ventral.

halves. A similar study is here reported on the incorporation of methionine labelled with S^{35}.

For each experiment early gastrulae, stage 10 of Shumway(2), of *Rana pipiens* or of *Hyla regilla* were removed from the membranes and bisected frontally along the animal-vegetative axis; the dorsal and ventral halves were separately collected and incubated in about 3 ml of sterile Holtfreter's solution containing 0.2 mg/ml radioactive methionine (Table I). The methionine was obtained from Abbott Laboratories, Chicago. As in the glycine study(1), efforts were made to prevent microorganisms utilizing the amino acid. The culture flasks were attached to open Warburg manometers and gently rocked in a water bath at 20°C for 5 hours. After incubation boiling 1M sodium acetate buffer at pH 4.5 was added to the flasks, the contents boiled, cooled and homogenized in a Potter type, ground glass homogenizer. The residue from centrifugation was washed at least 6 times with 10 ml aliquots of the acetate buffer. Ten mg of carrier dl-methionine was included in either the first or second wash aliquot. The residual protein was hydrolyzed with 25 ml of 8N HCl by autoclaving for 3½ hours at 15 lb. The general procedure outlined by Simpson and Tarver(3) was followed for the separation of methionine from other sulfur-containing amino acids, the conversion of methionine sulfur to sulfate, and subsequent determination of radioactivity and standard base equivalence of a benzidine sulfate precipitate. The data have been expressed in terms of specific activity, counts/min./μmole of sulfur. Self-absorption corrections have been made where necessary.

The results of 6 experiments with *R. pipiens* and 3 with *H. regilla* are shown in Table I. In every instance the dorsal halves had a higher specific activity. In *Rana* the average difference between the activities of the two halves was 14%, in *Hyla* 6%. Using a statistical method[†] for analyzing 4 paired measurements in *Rana*, the sigma of the difference of the means was 1.65 ($P < 0.01$). In experiments 3 and 4 the duplicate runs were averaged.

The methionine used in these experiments contained other radioactive compounds, namely: methionine sulfoxide and traces of homocystine and cystine, as shown by radioautographs of paper chromatograms of the fresh culture medium. In some experiments radioautographs were also made from chromatograms of the homogenate supernatant, of the hydrolyzed protein, and of the methionine fraction. No radioactive compound was found which was not present in the fresh culture medium.

Other experiments (not given in Table I) showed that, as in the glycine study(1), whole gastrulae had a much lower uptake of the amino acid than those bisected and that controls (gastrular halves boiled prior to incuba-

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[†] $\sigma d_M = \sqrt{\frac{Mx^2 - M^2x}{(n-1)}}$ where x = the difference between the members of the individual pairs.

tion or halves to which acetate buffer was added immediately after they were placed in the culture flasks) exhibited much less activity. Whole or cut embryos allowed to develop after 5 hours incubation in the culture medium suffered no apparent ill-effects of the treatment.

This study augments the evidence that the dorsal half of the amphibian gastrula has a greater physiological activity than the ventral half and specifically a higher rate of protein metabolism. Several studies by Brachet and recently that of Steinert(4) suggest a relationship between ribonucleoprotein and neural induction. The curve shown by the latter for the synthesis of ribonucleic acid resembles that which we obtained in our study of the uptake of glycine, a precursor of purine. We do not know whether the greater incorporation of the sulfur-bearing amino acid, methionine, by the dorsal half-gastrula than by the ventral half has peculiar significance. It is not impossible that this difference is indirectly related to the apparent importance of sulfhydryl groups in the induction of the neural plate by the organizer as shown by Rapkine and Brachet(5) and Lallier(6).

Summary. The dorsal half of the amphibian gastrula was shown to have a significantly greater incorporation of methionine into protein than the ventral half.

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Renal Excretion of Digitoxin in the Rabbit and Dog.* (19120)

SHIRLEY ST. GEORGE, RENÉ BINE, JR., MEYER FRIEDMAN, AND CATHARINE BLAND.
From the Mount Zion Hospital, San Francisco, Calif.

Although the renal excretion of digitoxin was found to be negligible in the rat(1), the normal human subject appeared to excrete as much as 40% of a given dose of the drug(2). In an effort to discover whether the rat handled digitoxin anomalously in this particular respect, the possible renal excretion of the drug was studied in two other species. The results of this study are here reported.

Methods. Three rabbits weighing an average of 2525 g were given 0.2 μg of digitoxin per g of weight by intravenous injection. The urines of these rabbits then were collected for 24 hours. The total urine volume was extracted for its digitoxin content according to previously described methods(1,2). The assay of the extract was performed with the embryonic duck heart preparation(1,2). Two male dogs weighing 9 and 10 kg respectively, were given 0.2 μg of digitoxin per g of body weight. A third dog weighing 15 kg was given 0.1 μg of digitoxin per g of body weight. The urine of each of the 3 dogs was collected for 24 and 48 hours. A 200 cc sample of each 24-hour urine was extracted and assayed exactly as described(2) for human urine. The extraction and sensitivity of the assay were such that a minimum of 10 μg could be detected and assayed in a 24-hour urine collection.

Results. The kidney of the rabbit does not appear to excrete digitoxin. Thus, although each of the 3 rabbits received approximately 500 μg of digitoxin by vein and each exhibited a good urine flow, none of the urine collections contained detectable amounts of digitoxin. This indicates that less than 0.5% of a given dose of digitoxin is excreted in an unchanged form by the rabbit kidney. It is of course

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* Aided by grants from The Life Insurance Medical Research Fund, Eli Lilly and Co., and the U. S. Public Health Service.

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TABLE I. Renal Excretion of Digitoxin in Dog After Its Injection (0.2 μg/g).

Dog	Wt, kg	Amt digitoxin given, μg	Urine (0-24 hr) vol., cc	Urine (0-24 hr) Digitoxin, μg	Urine (24-48 hr) Vol., cc	Urine (24-48 hr) Digitoxin, μg	% excreted
R-1	9	1800	900	18	450	N.D.*	1.0
R-2	10	2000	1400	35	400	5.	2.0
R-3	15	1500	2220	12	1980	N.D.	0.8

* Non-detectable.

possible, even probable, that a breakdown product of a glycoside might be expected.

The 3 dogs, however, were found to excrete a small amount of digitoxin during the first 24 hours. One of the 3 continued to excrete the drug the second day after its injection. As Table I indicates, the 2 dogs receiving 0.2 μg of digitoxin per g of body weight excreted 18 μg and 35 μg respectively the first 24 hours. The third dog, which received only 0.1 μg of the drug per g of body weight excreted 12 μg. One of the dogs (R-2) excreted 5 μg of the drug during the second 24 hour period of collection. Thus, the total renal excretion of digitoxin in the dog is about 1-2% of a given dose.

Discussion. The above results together with earlier studies(1,2) suggest that differences exist between various species in respect to renal excretion of digitoxin. Thus, whereas the rabbit appears to excrete no digitoxin in its urine, the dog excretes approximately 1-2%, the rat about 3%, and man as much as 40%.

Despite the relative paucity of digitoxin in the urine of the rat(1), rabbit, and dog, considerable amounts of the drug when given intravenously accumulate in the kidney of these animals(3). This is particularly so in the dog whose kidney, for several days after the injection of digitoxin, may contain far more digitoxin than is found in the 24 or 48 hours of urine collection. Moreover, this renal digitoxin is concentrated primarily in the cortical segment of the kidney. These observations suggest the possibility that the kidney of some mammals may destroy more digitoxin than it excretes. This does not appear to be so, however, in man.

Summary. The kidney of the rabbit was not found to excrete significant amounts of administered digitoxin. The dog appeared to excrete approximately 1-2% of an administered dose of digitoxin. The probability of the renal destruction of digitoxin in these species was discussed.

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Effect of Adrenal Hormones on Infection of Mice with Pneumoia Virus of Mice (PVM).* (19121)

J. MacLean Smith,† James S. Murphy,‡ and George S. Mirick.
(With the technical assistance of Miss Marjorie G. Huck.)
From the Department of Medicine, Johns Hopkins University School of Medicine.

The enhancing effect of urethane on the severity of the infection of mice with pneumonia virus of mice (PVM) was reported elsewhere(1). Because of certain striking similarities between the pharmacological effects of urethane and those attributed to adrenal cortical stimulation it seemed of interest to investigate possible adrenal effects on PVM infection. Murphy and Sturm(2) noted that the adrenal glands of rats given urethane were 40% heavier than controls. The effects of adrenal stimulation with adrenocorticotropic hormone (ACTH) or adrenal cortical substitution with cortisone have been summarized by Thorn *et al.*(3). Some similar effects of urethane are: Lympholysis(4), eosinopenia (5), suppression of inflammatory reaction(6), and fibroblast growth(7), chloride retention (8), and loss of potassium(9) and calcium (10), alkalosis(4), hyperglycemia(11,12), and increased gastric secretion(13).

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† Postdoctorate Research Fellow of the National Cancer Institute.

‡ Fellow, American Cancer Society.

In the present paper the results of treating mice with ACTH or cortisone and of adrenalectomy on PVM infection are described.

Materials and methods. Mice and virus. The O'Grady strain of Swiss mice and PVM strain 15 were employed throughout(1). Unless otherwise stated the mice weighed approximately 10 g each at the time of virus inoculation. *Titrations.* The usual methods (14) were used for performing virus titrations and calculating the 50% maximum lesion score (M.S. 50). Unless otherwise stated 20 mice were used in each virus titration. The method of Curnen and Horsfall(15) for performing hemagglutination tests was used. Virus was heated at 80°C for 10 minutes to eliminate the masking tissue component. *Drugs.* The ACTH was Actamour (Armour Laboratories). The cortisone was Cortone R (Merck). Dilutions of each drug were made in 0.85% saline so that the dose used was dissolved or suspended in 0.05 cc to 0.1 cc of fluid. The ACTH was stored, after dilution, at —20°C and the cortisone at room temperature. Each drug was given subcutaneously once daily. *Adrenalectomy.* Young mice weighing approximately 10 g were lightly anesthetized with ether. The adrenal glands were exposed and excised through a single mid-line dorsal skin incision and bilateral muscular incisions. The incisions were then closed with silk sutures and protected with collodion. Mice were maintained on subcutaneous injections of 0.5 cc of 1% glucose in 1.25% saline solution, daily from the time of operation until the end of the experimental period.

Experimental. Effect of ACTH on PVM infection. The effect of ACTH, on both the extent of the lesions resulting from PVM infection and the multiplication of the virus, was tested as follows: In 7 experiments simultaneous duplicate titrations of PVM were made in 2 matched groups of 20 mice weighing approximately 10 g each at the time of inoculation. In each experiment one group served as controls and each mouse in the other group was given a daily injection of ACTH. Twelve days after infection all surviving mice were sacrificed and the 50% maximum score (M.S. 50) was calculated. The results are shown in Table I (Exp. 1-7). It will be seen

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TABLE I. Comparison of PVM M.S. 50 Scores in Mice Receiving ACTH and Controls (Exp. 1-7) and Amount of Virus in Lungs at 6 Days (Exp. 8-11).

Exp. No.	Treated with ACTH Schedule	 M.S. 50 log	Control, M.S. 50 log	Difference from control, M.S. 50 log
1	(1)	4.21*	4.22	— .01
2	(2)	4.33	4.22	+ .11
3	(3)	4.24	4.61	— .37
4	(3)	4.79	4.57	+ .22
5	(3)	4.05	3.63	+ .42
6	(4)	3.70	3.63	+ .07
7	(5)	3.94	3.63	+ .42
	Avg			+ .10
8	(3)	5.60†	4.17	+1.43
9	(3)	6.53	6.18	+ .35
10	(3)	4.78	4.04	+ .74
11	(5)	4.93	4.04	+ .81
	Avg			+ .83

ACTH schedule	Daily injection	Total dose, γ/g
1	5 γ/g, D—1 to D+4; 20γ/g, D+4 to D+11	190
2	5 γ/g, D+4; 20 γ/g, D+4 to D+11	165
3	20 γ/g, D—2 to D+11	280
4	20 γ/g, D—2 to D+4	140
5	40 γ/g, D—2 to D+11	560

* M.S. 50 score at 12 days in mice treated with ACTH.

† M.S. 50 score at 12 days in normal mice inoculated with lung extracts from mice treated with ACTH.

that there is no significant difference in lesion scores in the two groups of mice. Since ACTH has been shown to suppress inflammatory reactions of various sorts(16), it seemed possible that the M.S. 50 score might not give the complete picture of ACTH effects on the infection. To test the possible effects of ACTH on virus growth other experiments were carried out. Four other matched groups of 8 mice each were inoculated with PVM 10^{-3}. Half of each group was given ACTH subcutaneously and the other half served as controls. Six days after virus inoculation the mice were sacrificed. The lungs from each group were pooled and extracted separately with trypticase soy broth (Baltimore Biological Laboratory) and the amount of virus in each pool was titered for infectivity in a fresh group of 20 mice. These results are also shown in Table I (Exp. 8-11). It will be seen that the lung extracts from the ACTH treated mice gave an average M.S. 50, 0.83 log higher than those from controls. To test the reproducibility of this type of assay, 5 other groups each comprising 4 normal mice were inoculated at the same time with PVM, dilution 10^{-3}. At 6 days the mice were sacrificed and the virus infectivity titer in pooled lung extracts from each group was measured in the usual fashion. The mean M.S. 50 was 6.61 logs and the standard deviation 0.061 log. For the purpose of this investigation a difference of 0.70 log or greater between control and experimental titers of virus in the lungs at 6 days is considered significant. Although the difference 0.83 log in virus titer in the lungs of ACTH treated and control mice is not great it is thought to be significant. It was decided to repeat the above experiments using cortisone rather than ACTH.

Effect of cortisone on PVM infection. Before testing the effects of cortisone on PVM infection it seemed important to determine, first, the toxicity of this substance. Following a single injection into 10 g mice, the M.L.D. 50% was found to be 400 γ/g. Mice which died showed pulmonary edema, vascular dilatation, and hemorrhage. Uninfected mice which received smaller sublethal amounts (15 γ/g) once daily for 6 days showed, on sacrifice at 12 days, no gross lesions in the lungs. However, the lung weight/body weight

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TABLE II. Comparison of PVM M.S. 50 Scores in Mice[*] Receiving Cortisone and Controls (Exp. 1-7) and Amount of Virus in Lungs at 6 Days (Exp. 8 and 9).

Exp. No.	Treated with cortisone: Schedule	Treated with cortisone: M.S. 50 log	Control, M.S. 50 log	Difference from control, M.S. 50 log
1	(1)	6.00[†]	3.63	+2.37
2	(2)	4.36	3.63	+ .73
3	(3)	5.00	3.73	+1.27
4	(4)	6.50	4.54	+1.96
5	(4)	5.34	4.82	+ .52
6	(5)	3.10	2.15	+ .95
7	(6)	2.59	2.15	+ .44
	Avg			1.18
8	(2)	5.59[‡]	4.04	+1.55
9	(2)	5.28	4.04	+1.24
	Avg			1.39

Cortisone schedule	Daily injection	Total dose, γ/g
1	50 γ/g, D—2 to D+11	700
2	50 γ/g, D—2 to D+4	350
3	25 γ/g, D 0 to D+11	300
4	12.5 γ/g, D 0 to D+6	87.5
5	9 γ/g, D 0 to D+6	63
6	5.6 γ/g, D 0 to D+6	39.2

[*] All mice were 3 to 4 wk old and weighed about 10 g each except those in exp. 6 which were 4 mo old and weighed 35-40 g and those in exp. 7 which were 6 mo old and weighed 40-50 g each.

[†] M.S. 50 score at 12 days in mice treated with cortisone.

[‡] M.S. 50 score at 12 days in normal mice inoculated with lung extracts from mice treated with cortisone.

ratio in these mice was 17% greater than in controls. This suggests that even these smaller doses of cortisone resulted in some pulmonary edema or congestion and is reminiscent of the findings in urethanized mice(1). The M.S. 50 scores observed when PVM was titered simultaneously in matched groups of mice, given cortisone and control, are shown in Table II (Exp. 1-7). The cortisone treated mice received 5-50 γ/g of the hormone subsubcutaneously once daily. In the same table (Exp. 8, 9) are recorded the M.S. 50 scores of virus in the lungs of cortisone treated and normal control mice tested at 6 days after inoculation with PVM 10^{-3}. These mice received 50 γ/g of cortisone subcutaneously once daily. It will be seen that the average lesion score in cortisone treated mice was 1.18 logs greater than in controls and that the amount of virus at 6 days was also greater in the cortisone treated mice by 1.39 logs. The amount of virus at 2, 4, 6, and 8 days after inoculating PVM 10^{-3} into normal, ACTH treated and cortisone treated mice, was also tested by the capacity of heated lung extracts to agglutinate mouse erythrocytes. The results are recorded in Fig. 1. It is seen that none of the heated extracts of lungs removed 2 days after virus inoculation caused hemagglutination. At 4, 6, and 8 days the average titer in ACTH treated mice was about equal to that in controls but in cortisone-treated mice was about 1 log greater than in the controls. This, again, is similar to the finding in mice which were treated with urethane(1).

Effects of adrenalectomy on PVM infection. Since both ACTH and cortisone treatment seemed to result in increased amounts of PVM in the lungs of mice at 6 days after virus inoculation, and the latter also accentuated the lesions observed at 12 days, it seemed of interest to test the effects of adrenalectomy on PVM infection. In three experiments the adrenal glands were removed from each of 24 10 g mice. Seven to 10 days after operation PVM was titered in the usual fashion in 20 of the mice. Simultaneous duplicate titrations were done in intact control mice of the same age. Eight additional mice, 4 adrenalectomized and 4 intact, were inoculated in each experiment with virus dilution 10^{-3}, sacrificed at 6 days and extracts of their pooled lungs compared for infectivity. The results are shown in Table III. It will be seen that the average

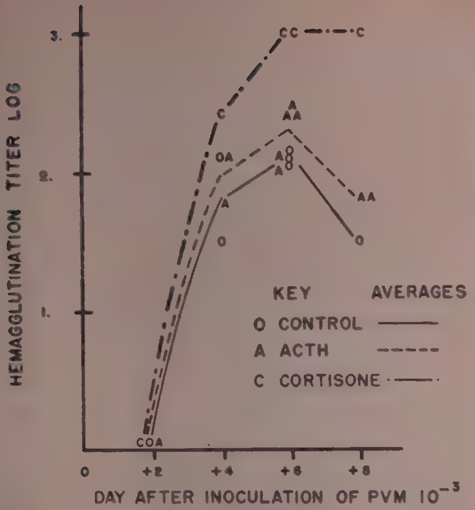

FIG. 1. Hemagglutination titers of heated lung extracts from mice treated with ACTH or cortisone and controls. Most mice died on the 7th or 8th day after virus inoculation.

M.S. 50 score in the adrenalectomized mice was slightly (0.33 log) less than in controls. The average titer of virus at 6 days was 0.89 log less in the adrenalectomized mice. These differences are not great but are consistently in the opposite direction from the findings in mice which received ACTH or cortisone. Taken together the findings strongly suggest that adrenal stimulation and especially cortisone administration enhances PVM infection and adrenal extirpation has the opposite effect.

Effects of ACTH and cortisone on pulmonary tumors. The similar effect of ACTH, cortisone and urethane on PVM infection suggested that prolonged treatment of mice with these drugs should be tested for possible oncogenic action. Twenty-five mice one month old were injected thrice weekly with 400 γ of ACTH for 3 to 5 months. Forty-three other mice were similarly treated thrice weekly with 250 γ of cortisone. These mice and 110 untreated controls were sacrificed at 6 months. The tumor incidence in the ACTH and cortisone treated groups was 36% and 25%, respectively, as compared with 31% in the controls. It is concluded that ACTH and cortisone in the doses tested had no oncogenic effect.

Discussion. The effects of ACTH and cortisone have been tested in a variety of bacterial infections(16); in general, treatment with these drugs has resulted in some suppression of the inflammatory reaction but enhancement and dissemination of the infection. The influence of ACTH on certain virus infections of man has been reported. Finland *et al.*(17) described the symptomatic improvement of a patient with primary atypical pneumonia treated with ACTH. However, there was no apparent effect on the course of the disease. Treatment with ACTH may have alleviated the severity of acute viral hepatitis (18), but this drug did not influence the course of poliomyelitis in human beings(19) when given after the onset of symptoms

A number of reports have appeared recently concerning ACTH and cortisone effects on experimental virus infections in laboratory animals. ACTH has been found to have little or no influence on experimental infections in mice with influenza virus(20,21), the Lansing

TABLE III. Comparison of PVM M.S. 50 Score in Adrenalectomized and Control Mice (Exp. 1-3) and Amount of Virus in the Lungs at 6 Days (Exp. 4-6).

Exp. No.	Adrenalectomized, M.S. 50 log	Control, M.S. 50 log	Difference from control, M.S. 50 log
1	2.69	3.38	—.69
2	2.63	2.67	—.04
3	3.17	3.43	—.26
	Avg		—.33
4	4.14	5.05	—.91
5	3.39	4.16	—.77
6	4.96	5.95	—.99
	Avg		—.89

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strain of poliomyelitis virus(22,23), or the virus of western equine encephalomyelitis(23). Ainslie *et al.*(24) reported that large doses of ACTH in monkeys seemed to make them more susceptible to infection with the Minnesota strain of poliomyelitis. Cortisone has been shown to markedly enhance poliomyelitis infection in mice and hamsters(22). It increased the susceptibility of old mice to Coxsackie virus(25), of mice to influenza virus (26), and to West Nile, Ilheus and Bunyamwera viruses(27), and of guinea pigs to vaccinia virus(28). Moreover, cortisone treatment of embryonated eggs has resulted in significantly greater concentrations of both influenza and mumps viruses in the allantoic fluid(29).

Our findings that ACTH, in the doses employed, produced little effect on PVM in mice whereas cortisone treatment resulted in rather striking increase in the severity of the infection are in general agreement with the findings reported in other virus infections. It is quite possible that the ACTH employed may have lost potency through storage and that larger dosage or more frequent treatment might have resulted in effects similar to those of cortisone. The mechanism through which this effect takes place is obscure but the evidence presented in this paper and elsewhere(1) suggests that the enhancing effect of urethane on PVM infection may work through a similar mechanism.

Summary. The treatment of mice with ACTH did not influence their susceptibility to PVM infection but slightly enhanced virus multiplication. Cortisone treatment enhanced both the virus infection and the multiplication of virus to a greater degree. Adrenalectomy rendered mice slightly less susceptible to infection and retarded virus growth.

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Growth Studies on *Polytomella agilis*. (19122)

P. A. LITTLE, J. J. OLESON, AND J. H. WILLIAMS.
From Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

The nutrition and metabolism of phytomonads and related species have been studied by several investigators. Doyle(1) has reviewed the problem of physiological classification of protozoa on the basis of their known nutrition. That ethanol and acetate are utilized by various species has been known for many years(2,3). A number of green and colorless flagellates such as Euglena, Astasia, Polytoma, Polytomella, Chlorogonium, and Hyalogonium have been found to be unable to utilize sugars and hence are dependent on simple acids and alcohols. Lwoff, Ionesco, and Gutmann(4) showed that *Polytomella caeca* lacks the hexokinase required for utilization of exogenous sugars. Albaum *et al.*(5) investigated this problem in Euglena.

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Experimental. This report deals with the growth responses of an organism which we have isolated and identified as *Polytomella agilis*. This organism was freed from bacteria by cultivation in media containing 50 μg per ml of aureomycin, penicillin, and sulfadiazine. It was first grown in a complex, defined medium supplemented with any one of a number of concentrated fruit extracts, the active principle of which proved to be ethanol. Modified from a medium developed for *T. cruzi*(6) this medium was far more complex than necessary. The organic constituents were as previously reported(6). It was found that substances such as glucose, levulose, and hexose diphosphate are not utilized by *P. agilis*. Substances such as glutamine, adenine, creatine, guanosine, nucleic acid, uracil, and uric acid were not essential but were found to be readily utilized as nitrogen sources. Urea was inactive. While the various constituents of the medium were not rigorously purified to eliminate trace elements, it was possible to demonstrate a requirement for magnesium and calcium. Cultures maintained by weekly transfer in this basal medium supplemented with 0.2% ethanol were used to inoculate sets of 5 tubes containing 10 ml volumes of the medium supplemented with various substances tested for ability to replace ethanol. The inoculum was 0.1 ml and the densities, measured turbidimetrically, reached their peak in 4 to 5 days. Cultures having density of "30" were found able to initiate growth when diluted 1-100,000.

Substances utilized for carbon-nutrition. Seven substances stimulated growth of this organism: Ethanol, butanol, sodium acetate, ethyl acetate, *n*-butyl acetate, sodium propionate, and sodium butyrate. The optimum range of activity of these substances was 0.1-0.2%. Ethanol, sodium acetate, and butanol were tested at several levels. The minimum requirement for ethanol was found to be around 0.03%, and the minimum requirement for sodium acetate and butanol appeared to be nearer 0.04%. It seems interesting to note that cultures maintained on 0.03-0.04% of ethanol remained at peak density for only 2 or 3 days whereas cultures maintained on 0.06 through 0.2% were active for successively longer periods up to 2 weeks. Ethanol was the only substance well tolerated at the 0.4% level. The organism tolerated concentrations of ethanol up to 2%. The utilization of propionate by our organism is of interest in view of the problem presented by odd-numbered carbon acids. Huennekens *et al.*(7) have investigated the role of the liver cyclophorase system in the complete oxidation of propionate.

Substances found inactive. Thirty-two substances were found unable to replace ethanol in our tests. They are: Formic acid, methanol, acetaldehyde, sodium oxalate, acetone, allyl alcohol, glycerol, isopropyl alcohol, lactic acid, malonic acid, methyl cellosolve, propylene glycol, sodium citrate, cellosolve, malic acid, succinic acid, amyl alcohol, diethyl ketone, mannitol, methyl amyl alcohol, pentanol-3, *n*-propyl acetate, glucose, butyl cellosolve, dulcitol, diacetone alcohol, inositol, methyl isobutyl ketone, sorbitol, triethanolamine, ethyl butyl ketone, heptanol-3. The inactivity of acetaldehyde is interesting in view of the studies of Stadtman and Barker (reviewed by Krampitz)(8) on the conversion of ethanol and acetate through acetaldehyde to fatty acids by *Clostridium kluyveri*.

TABLE I. Composition of Simplified Basal Medium.

	Amt per liter
Na_2HPO_4	.5 g
KH_2PO_4	.5
$MgSO_4 \cdot 7H_2O$	3.75 mg
$MgCl_2 \cdot 6H_2O$	20
$CaCl_2 \cdot 2H_2O$	18.75
$FeCl_3 \cdot 6H_2O$	.3
$MnCl_2 \cdot 4H_2O$	.01
$ZnCl_2$	.01
Glutamine	100
Thiamine	1
Vit. B_{12}	6 μg

Final pH: 6.8

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TABLE II. Growth Responses of *Polytomella agilis*.

Medium	Carbon source, .2%	Avg photodensity readings (5 tubes) 1 day	3 day	6 day	8 day	10 day	12 day	Final pH
Simplified basal (Table I)	Ethanol	6	43	63	48	45	37	5.96
	Acetate	5	18	43	56	49	42	7.91
	Butyrate	5	29	63	63	60	52	7.81
Same minus B_{12}	Ethanol	7	24	37	37	37	28	6.37
	Acetate	5	14	24	29	32	32	8.48
	Butyrate	6	16	31	40	40	40	7.73
" " thiamine	Ethanol	7	33	43	41	43	40	3.31
	Acetate	4	34	48	49	43	37	8.23
	Butyrate	5	30	38	46	50	50	7.24
Same minus both B_{12} and thiamine	Ethanol	5	18	28	29	31	31	5.00
	Acetate	6	18	30	33	32	34	8.40
	Butyrate	7	16	31	40	41	40	7.82
Complex basal (51 organic constituents)	Ethanol	3	25	36	37	38	35	6.21
	Acetate	3	12	24	31	31	33	8.16
	Butyrate	2	20	42	46	45	43	7.91
	None	6	8	8	8	8	8	6.90

Albaum *et al.*(5) observed that malic and succinic acid are utilized by Euglena.

Experiments with a simplified basal medium. The medium described in Table I was developed as the result of a study of the effect of eliminating various constituents from the more complex medium. It will be noted that glutamine provides the only source of nitrogen; 100 mg per liter supported optimum growth. Under the conditions of our tests, neither glutamic acid, aspartic acid, nor glycine gave as good growth as glutamine or asparagine.

In contrast to our medium, Lwoff *et al.*(4) employed a medium for *Polytomella caeca* consisting of 1 g per liter of ammonium sulfate, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.5 g KH_2PO_4, 1 g sodium acetate, 3 ml ethanol, 0.01 mg thiamine, and 10 mg ferric citrate. They observed a requirement for thiamine but not for vit. B_{12} which is important in the nutrition of Euglena(9). Our organism grew poorly in this medium even when supplemented with vit B_{12}.

As shown in Table II, omission of thiamine and vit. B_{12} from our basal medium caused retardation of growth of our organism in the presence of ethanol, acetate or butyrate. The effect was more marked with vit. B_{12} than with thiamine. However, serial subcultures were not made to determine the absolute requirement of these vitamins.

Of particular interest are the final pH values obtained in each instance. Lwoff *et al.* (4) observed that growth of *Polytomella caeca* in the presence of ethanol leads to a marked acidification of the medium due presumably to the uncompensated liberation of $SO_4^{=}$ from ammonium sulfate, and that growth in the presence of sodium acetate leads to a marked alkalinization of the medium which they attributed to the liberation of sodium ions. In Lwoff's experiments this problem was met by using a mixture of ethanol and acetate.

The effect of thiamine and vit. B_{12} on acid production on the ethanol medium is shown by the pH readings in Table II. There is a slight drop in pH when both thiamine and vit. B_{12} are omitted. The omission of only thiamine results in a marked pH drop. This indicates that vit. B_{12} stimulates the production of acidic metabolites, which are not metabolized in the absence of thiamine. In the presence of acetate or butyrate, an alkaline pH is obtained which masks the effect of the two vitamins.

Polytomella agilis has been maintained 4 months by weekly subcultures on the simplified basal with 0.2% ethanol. *Euglena gracilis*, *E. gracilis var. bacillaris*, and *Parapolytoma satura* have also been successfully prop-

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agated in this medium for varying lengths of time.

Summary. The nutritional requirements of a strain of *Polytomella agilis* were investigated. Ethanol, butanol, acetate, propionate or butyrate were utilized as carbon sources. Glutamine was adequate as a sole source of nitrogen. Thiamine and vit. B_{12} were stimulatory. A simplified medium, adequate for continuous subculture of this organism, is presented.

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Effects of Adrenalectomy on Blood Cells of Mice with Special Reference to Eosinophiles.* (19123)

KATHRYN F. STEIN AND CYNTHIA MARTIN.

From the Department of Zoology, Clapp Laboratory, Mount Holyoke College

Numerous references to the effects of adrenalectomy on the numbers of blood cells occur in the literature. The various investigators tend to agree that total removal of both adrenals results in an anemia which is manifested by decreases in total red cell count and in Hb values, provided hemoconcentration of the blood is prevented by supplying the salt factor(1-5). The situation is less clear with regard to the leucocytes. The results reported range from a decrease through little or no change to a significant increase, in total white count, in lymphocytes, and/or in neutrophils(1-13). Either there has been no consideration of eosinophilic leucocytes apart from neutrophilic or the statement has been made that the former seem unchanged(2,3,10).

In the course of a series of experiments with adrenalectomized animals some data were accumulated with regard to the white blood cell picture before and at various intervals after the operation(14) and later additional counts were made. Special attention has been given to variations in the numbers of eosinophils and bone marrow has been examined to determine the effects of adrenal insufficiency upon the myeloid-erythroid ratio and upon mitotic division.

Material and methods. All counts included in this report were made on 56 young male adult mice, hybrids of mixed ancestry, raised and maintained in wire cages in a warm animal room (72-78°F) and fed Purina dog chow supplemented with occasional milk and lettuce. Following adrenalectomy they were kept in wooden boxes and given 0.9% NaCl in tap water for drinking. Bilateral adrenalectomy was performed under light ether anesthesia following an intraperitoneal injection of 0.1 cc of 1 part Nembutal to 20 parts 0.9% NaCl solution. A subcutaneous injection of 5 cc of salt solution was given postoperatively.

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The method of Speirs and Meyer(15) was used to test for completeness of the operation, and only data from those mice which were completely adrenalectomized, as evidenced by no change or a rise in the number of eosinophiles after an injection of epinephrine, have been included. Total white counts were done in the usual manner. Differential counts of at least 200 cells were made from smears stained in May Grünwald Giemsa solutions for 31 of the mice after adrenalectomy and for all except 6 prior to adrenalectomy. Data for the latter, and for 25 of the adrenalectomized animals, are from direct differential counts. The number of eosinophils was determined according to the direct method of Speirs and Meyer(15); polymorphonuclear were distinguished from mononuclear cells by use of a 15x ocular in the course of making the total count in the Neubauer counting chamber, and the figures for the neutrophils were then obtained by subtracting the number of eosinophils from the number of polymorphonuclear cells per cu mm. A comparison of test counts on 5 mice by both methods indicated very little variance for neutrophils or mononuclear cells but a wider range and higher values for eosinophils by the smear technic. Intervals of 1 to 30 days elapsed between adrenalectomy and time of counting and data have been arranged in four series on this basis: 1-2, 4-7, 9-13, and 17-30 days. Counts were done on some of the mice at only one interval, on others at 2 or 3; but at least 3 days elapsed between successive counts on individual mice. Bone marrow smears were made directly, with the use of a hemostat, from the longitudinally split femur or sternum. Femoral smears were treated by the Feulgen method(16), those from the sternum were stained in May Grünwald Giemsa solutions. One thousand cells per smear of femoral marrow were differentiated as belonging to the myeloid or erythroid series according to the method recommended by Polli(17), and the M/E ratio computed. The number of mitotic figures (chromosome stages only) per thousand cells was estimated on the same smears in most cases; for a few mice in each category, operated and intact, sternal smears were used. Comparative counts on smears from the same animals by both methods showed little or no variation. Reticulocytes were counted by the method of Friedlander and Wiedemer(18) with slight modifications.

Observations and results. Since the mice were of different generations from mixed ancestry a good deal of variation was expected between blood counts of individual animals. The effect of adrenalectomy on the leucocyte count showed the same trend in most cases, however, whether the count before operation was high or low. The picture, except at the 1-2 day interval, was characterized by a leucocytosis (Table I) for which the increased numbers of lymphocytes were mainly responsible. Absolute numbers of eosinophils were also higher. The behavior of the neutrophils was variable. The average numbers of the various types of cells at each interval after adrenalectomy, with the exception of 1-2 days, illustrate these trends and also show the increase in degree of effect with increase in length of interval after operation (Table I). The differences in numbers of mononuclear cells and eosinophils are significant within each of the 3 later series. Computations were made by Student's t test(19) and t values were higher than those expected at the 1% level. This was not true for the neutrophils; even the greatest difference, at the 4-7 day interval, was one which might be expected more than 5 times in 100 on the basis of chance alone. For all computations a comparison was made between counts of the same individuals before and after operation within each series; some of the mice are represented by counts in 2 or 3 different series. Lymphocytosis occurred after adrenalectomy in each one of the latter and only one failed to exhibit an eosinophilia. Of the 28 individuals counted at only one interval, 6 had fewer lymphocytes and 2 showed a slight eosinopenia after

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TABLE I. Average No. of White Blood Cells Before and After Adrenalectomy.

Type of count	No. of mice	Count before adrenalectomy	Interval, days	Count after adrenalectomy	Increase in No.	Stand. error	Increase, %
Total	10	11920	1-2	11340	–580		–5
	35	9280	4-7	12394	3114		33
	25	9808	9-13	13673	3865		39
	12	9758	17-30	17140	7382		76
Mononuclear	10	10350	1-2	10185	–165		–2
	35	7708	4-7	10334	2626	697	34
	25	7635	9-13	10840	3205	734	42
	12	7642	17-30	14739	7097	1950	93
Eosinophil	10	165	1-2	103	–62		–37
	35	94	4-7	160	66	24	70
	25	138	9-13	275	137	33	99
	12	129	17-30	312	183	42	141
Neutrophil	10	1405	1-2	1053	–352		–25
	35	1478	4-7	1900	422	233	29
	25	2035	9-13	2558	523	478	26
	12	1988	17-30	2089	101		5
Total	56	9600					
Mononuclear		7940					
Eosinophil		110					
Neutrophil		1550					

operation. These were from the 4-7 day series with the exception of one mouse from the 9-13 day group.

Consideration of the 1-2 day series (Table I) illustrates the importance of counting the same individuals before and after operation if accurate results are to be obtained. If a comparison of the average counts for the 12 mice in this series is made with those for all 56 mice before adrenalectomy there appears to be an increase in total count and in each type of cell except eosinophils. Actually for this particular group average decreases in all 3 types of leucocytes occurred at this interval after operation. These are obviously not significant with the possible exception of that for the eosinophils. As noted previously there is great variation in the neutrophil counts but none of the increases noted in this type of cell after adrenalectomy is significant. There is certainly no evidence of a neutropenia resulting from the operation, at least in the average counts. This is true irrespective of the method of counting and of whether the mice had had previous injections of epinephrine or not. A comparison of the percentage increases for the total count and for each type of leucocyte indicates that those for the mononuclear cells closely parallel those for the total count at the various intervals (Fig. 1). The eosinophils show by far the greatest average per cent increases.

An attempt was made to trace the origin of the increased numbers of leucocytes by a study of bone marrow and lymph node tissue. No real difference was observed from examination of stained imprints of the latter but some variations were noted in the former. The average number of mitotic figures, including late prophase (chromosome), metaphase, anaphase and telophase stages prior to reconstruction, was 5.5 ± 0.4 per thousand cells in the femoral marrow of 18 intact mice while for 25 animals adrenalectomized for from 8 to 30 days this was reduced to 3 ± 0.24 per thousand. This was a 45% decrease and the difference was found to be significant (5 X S.E.). The myeloid/erythroid ratio was also altered significantly after an 8-18 day absence of the adrenal glands. The variation in 10 intact mice was from 0.5/1 to 0.8/1, average 0.7/1; and in 14 adrenalectomized from 0.7/1 to 1.8/1, average 1.2/1. This average increase of 0.5/1 could indicate either a relative increase in myeloid or a relative reduction in erythroid cells. Since the number of reticulocytes is considered an index of bone marrow activity, counts were made on 18 adrenalectomized mice (from the 4-5 and 9-13 day series) and compared with data from the same number of controls. The average number of reticulocytes per 1000 red cells was

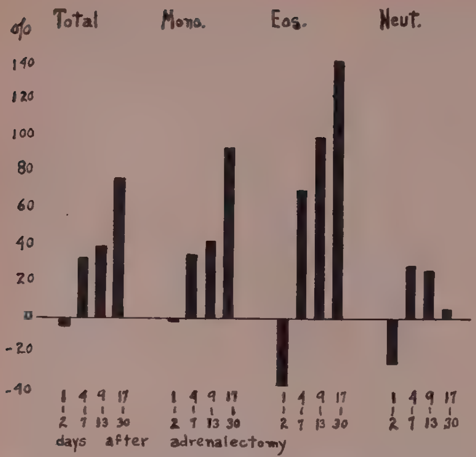

FIG. 1. % changes in absolute numbers of leucocytes at various intervals after adrenalectomy.

53 ± 4 for the former and 31 ± 2 for the latter, a 66% increase after adrenalectomy and a significant difference (4.8 X S.E.). No consistent variation was observed between degree of reticulocytosis and length of interval.

Discussion. The reasons for the difference in results obtained in investigations of the effect of adrenalectomy on circulating white blood cell values(1-13) are not apparent. Infection(8), individual reaction(12), and feline agranulocytosis(13) have been suggested. Other factors which might be involved in different instances are incompleteness of operation, length of the interval between operation and counts, small numbers of animals, and comparison of one group of adrenalectomized animals with a different group of controls rather than of counts from the same subjects pre and postoperatively. The results of the present experiment are in agreement with those of White and Dougherty(4) in showing increases in the total number of leucocytes and in the absolute number of mononuclear cells. As in their data, increases were progressively greater with length of interval after 2 days. However, counts were made at more intervals and over a longer period (2-30 versus 2-6 days) in the present study. White and Dougherty (4) reported an increase in polymorphonuclear leucocytes in their adrenalectomized mice which received salt treatment and a decrease of these cells in untreated animals. Neither a neutropenia nor a significant neutrophilia was observed in our mice but there was a significant and progressive increase in the absolute number of eosinophils after adrenalectomy. Reports in the literature either do not give data on eosinophils or state that no significant change occurred.

Examination of the bone marrow would be expected to aid in the interpretation of the mechanism of action of the adrenal hormones on the processes of erythropoiesis and myelopoiesis. On the basis of the decreased number of polymorphonuclear cells in the blood of adrenalectomized mice which were not salt treated, White and Dougherty(4) suggested the likelihood of an actual suppression in the production or delivery of these cells. Hypoplasia of the marrow of adrenalectomized cats was noted by Lewis(1). An increased myeloid/erythroid ratio was observed in this investigation in mice, and by Piliero *et al.*(5) in rats, after adrenalectomy. In conjunction with a hypoplastic marrow such an increased ratio might represent only a relatively greater inhibition of erythroid than of myeloid elements rather than a stimulation of the latter. A recent investigation(20) points to the stimulating effects of cortisone on myeloid tissue and thus lends support to the suggestion of inhibition resulting from lack of the hormone. Pilero *et al.*(5) comment on the increased level of *young* neutrophilic forms in the marrow of adrenalectomized rats and the reduction of this immaturity by Compound E. Increase in the number of young versus older myeloid cells was not observed in our mice but there was a significant decrease in the number of mitotic figures in the bone marrow of the adrenalectomized animals, indicating a reduction in hemopoietic activity.

As regards erythropoiesis the percentage of reticulocytes in the circulating blood is considered an index of bone marrow activity. The increased proportion of these cells observed by us and by previous investigators(2,5,12) after adrenalectomy might thus be interpreted as indicating a more than normally active marrow. This is not in agreement with the observations noted above. An increased rate

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20. Quittner, H., Wald, N., Sussman, L. N., and Antopol, W., *Blood,* 1951, v6, 513.

of maturation and release of reticulocytes would also account for the data but it seems questionable to assume that adrenalectomy would increase the degree of immaturity in leucocytes and hasten maturation of erythrocytes. Another, and seemingly a more likely, possibility would be a decreased rate of maturation of the circulating reticulocytes thus allowing for their accumulation in the blood.

Summary. Adrenalectomy resulted in an increase in total count and increased absolute numbers of mononuclear and eosinophilic cells in the circulating blood of adrenalectomized mice. The increases were progressively greater with increases in the length of the interval after adrenalectomy from 4-5 days to 17-30 days. There was no consistent effect on neutrophiles. Reticulocyte percentages increased in the circulating blood. The number of mitotic figures in the bone marrow was reduced, while the ratio of myeloid to erythroid elements was increased. All the differences noted were found to be significant. The results suggest a decreased rate of activity and of maturation of cells in the bone marrow and an increase in the length of period of maturation and longevity in the circulating blood.

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Toxicity Studies on Subtilin. (19124)

Robert H. Wilson, Floyd DeEds, and Lelland J. Rather.

From the Pharmacology Laboratory, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture, Albany, Calif. and the Department of Pathology, Stanford University School of Medicine, San Francisco.

The possibility that it may become desirable to use antibiotics in the normal diet either for growth promotion or as food preservatives requires that adequate knowledge be available on their long-term effects. The present study dealing with the continued feeding of subtilin-containing diets to rats was prompted by the observation that subtilin appeared to have possibilities as a food-preserving substance(1).

Wilson, Humphreys, Reynolds and Lewis (2) have reported on the absorption of subtilin in acute experiments and commented on accompanying toxic reactions. Consideration of that data and the *in vitro* studies of others (mentioned below) leads to the presumption that chronic oral ingestion would be harmless. Subtilin is a polypeptide(3) inactivated and presumably hydrolyzed by proteolytic enzymes(4). Destruction in the gastrointestinal tract could be expected. In the acute toxicity studies(2) it was mentioned that a single rabbit received 1 g of subtilin/kg by stomach tube. After 5 hours only 2 μg were found in the urine, 15 mg in the intestine and 0.5 g in the stomach, or a total of 13% of the administered 4 g. The highest blood level was 0.1 ppm. This would indicate rapid inactivation or destruction of the subtilin entering the intestine. Subtilin is but sparingly soluble in physiological saline solutions and in body fluids(4). This insolubility accounted for all observed acute toxic effects* and might limit chronic oral toxicity. Andersen and Michener(1) suggested that "although *B. subtilis* is often found in foods, where it may well have produced appreciable quantities of subtilin, no cases of food poisoning have ever been attributed to it." They suggested further that the predominant intestinal microflora would not be adversely affected since subtilin is not active against Gram-negative bacteria:

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1. Andersen, A. A., and Michener, H. D., *Food Technology*, 1950, v4, 188.

2. Wilson, R. H., Humphreys, E. M., Reynolds, D. M., and Lewis, J. C., Proc. Soc. Exp. Biol. and Med., 1949, v71, 700.

3. Jansen, E. F., and Hirschmann, D. J., *Arch. Biochem.*, 1944, v4, 297.

4. Dimick, K. P., Alderton, G., Lewis, J. C., Lightbody, H. D., and Fevold, H. L., *Arch. Biochem.*, 1947, v15, 1.

Methods. The rats were weanling male albinos of the strain used in this laboratory and previously described(5). The animals were caged in groups of 5, furnished water and food *ad libitum,* and body weights and food intakes were determined weekly. The subtilin was a refined sample obtained from the Western Regional Research Laboratory. It was ground to a powder in a mortar and then mixed with successive additions of basal diet, first in the mortar and finally in a mixing machine. Diets with lower concentrations of subtilin were made by serial dilutions of the most concentrated diet with basal diet. All subtilin-containing diets were refrigerated until offered to the animals. The basal diet was the one usually used in this laboratory for stock animals and for continued feeding experiments. It consisted of: yellow cornmeal 73%, linseed oil cake meal 10%, alfalfa meal 2%, casein 10%, cod-liver oil 3%, bone ash 1.5%, and sodium chloride 0.5%. The diets fed contained 0, 0.0062, 0.0125, 0.025, 0.05 and 0.1% of subtilin. The highest level (0.1%) is 100 times the concentration used by Andersen and Michener(1) in their original experiments. Each diet was fed to 5 animals, with 10 rats serving as controls, for 220 days. At the close of the feeding period the rats were autopsied; the organs were weighed and representative sections of tissue were fixed in 4% formaldehyde. Paraffin sections stained with hematoxylin and eosin were prepared for histopathological examination.

Results. The appearance and activity of the animals were normal throughout the course of the experiment, except for those suffering with respiratory infection. The incidence and severity of these infections were no greater than in the colony as a whole. An epidemic in the colony of fulminating bronchopneumonia led to an earlier termination of the experiment than was originally planned, to avoid possible loss of the animals through this disease.

Growth of the rats was not adversely affected. There was a definite suggestion of augmented growth in most of the groups receiving subtilin. For example, after 190 days, animals receiving diets containing 0.1, 0.05 and 0.0125% subtilin averaged 25 to 30 g heavier than their controls. Because of individual variations within the small groups, the differences in body weight were not shown to be statistically significant. The possibility of more rapid growth should be checked carefully, since there are reports that oral ingestion of certain antibiotics increases the rate of growth of chicks(6) and pigs(7).

At autopsy, organs appeared normal except for a few instances, scattered among the groups, of partially consolidated lungs, and one hyperemic kidney in a control animal. Weights of the organs did not differ significantly from normal, although there was a suggestion of increased weight of adrenals and testes. The gross observations were confirmed by the microscopic examination, with no morphological changes attributable to the subtilin being observed. Tissues studied in this manner included liver, kidney, spleen, pancreas, adrenal, testis, heart, lung and thyroid.

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* In our earlier paper(2) on absorption and acute toxicity of subtilin, gross evidence was given of deposition in the tissues of precipitated subtilin, with formation of chronic abscesses if the deposits were in the subcutaneous or muscular tissues. Histopathological examination of some of these lesions now has been made. That precipitation of subtilin at the site of injection or in the blood stream does occur is indicated by the following examples: In a rabbit, injected intramuscularly 24 hours earlier, polynuclear cells, many of which were eosinophiles, infiltrated widely into the muscle. In one zone the muscle fibers were displaced by clumps of eosinophilic material showing some irregularity of staining and with finely-basophilic strand-like threads scattered throughout. Five weeks after subcutaneous injection in a guinea pig, the cavity of the resulting abscess was bordered by a wall about 2 mm thick, composed of closely packed multinucleated giant cells of the foreign body type. Many of these contained clumps of amorphous, homogeneous, eosinophilic material which evidently was the source of the persistent reaction. In a rabbit which was killed by the intravenous injection of 50 mg of subtilin/kg, the right heart was filled, the left heart was empty and the lungs were pale. Microscopic examination showed the right heart to be filled with red cells and irregular masses of homogeneously eosinophilic material. The large and small vessels of the lung were distended, as if by an injection mass, by homogeneously eosinophilic material of similar nature.

5. Wilson, R. H., De Eds, F., and Cox, A. J., Jr., *Cancer Research,* 1941, v1, 595.

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Conclusions. The feeding to rats of diets containing as high as 0.1% of subtilin for 220 days was not detrimental as judged by growth, activity, organ weights and macroscopic and microscopic appearance of the tissues. The previously reported evidence for precipitation of parenterally-administered subtilin has been substantiated by microscopic examination.

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Hemagglutination Reaction in Streptococcal Infections and Acute Rheumatic Fever.* (19125)

WILLIAM M. M. KIRBY. (Introduced by R. H. Williams.)

From the Department of Medicine, University of Washington School of Medicine, Seattle, Wash.

A hemagglutination test described by Middlebrook and Dubos(1) is being widely studied in tuberculous infections in experimental animals and in man. Preliminary evidence indicates that this reaction has greater specificity than other serological procedures such as the complement fixation test, and that it tends to be positive only during active tuberculous infections(1,2).

These promising results suggested the possibility that hitherto undetected antibodies might be discovered during the active phase of rheumatic fever with the hemagglutination technic. The present study was undertaken, therefore, to determine whether various streptococcal extracts or products could be adsorbed onto sheep erythrocytes, and to observe the behavior of the sensitized cells in the presence of sera from patients with streptococcal infections and acute rheumatic fever.

Materials and methods. The following streptococcal filtrates and extracts were prepared for hemagglutination tests: A. *Heated filtrate of a culture of whole streptococci.* A Group A, non-typable streptococcus recently isolated from a patient with acute rheumatic fever was grown overnight in 500 cc of Todd-Hewitt broth. The culture was placed in a boiling water bath, and the total volume was reduced from 500 cc to 100 cc by evaporation. Following Seitz filtration, the filtrate was diluted 1 to 6 in normal saline for attempts at sensitization of sheep erythrocytes. When negative results were obtained, the filtrate was further concentrated from 100 cc to 20 cc by evaporation. Even when diluted 1 to 6 this thick, syrupy material was unsatisfactory for sensitization because it caused hemolysis of most of the red cells. B. *Hydrochloric acid extract.* The streptococcus used was the same as for preparation A. The crude streptococcal extract, containing both the group-specific C substance and the type-specific M antigen was prepared in the usual fashion(3) by boiling the organisms with HCl at a pH of 2.5, discarding the cellular residue, and neutralizing the supernatant. The extract was diluted 1 to 4 in normal saline for attempts at sensitization. C. *Formamide extract.* The method of Fuller(4) was used to extract the C substance. The streptococcus was the same as that used for preparations A and B. For sensitization the extract was diluted 1 to 4 in saline. D. *Streptolysin O.* Streptolysin O was prepared from the Richards strain according to the method of Rantz and Randall(5), and was stored in the refrigerator in the oxidized state.

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TABLE I. Antistreptolysin and Hemagglutination Titers of 8 Patients with Non-Streptococcal Upper Respiratory Infections, and of 7 Patients with Acute Rheumatic Fever.

			Sensitizing antigen	
	Patient	Antistreptolysin titer (units/cc)	Formamide extr.	Streptolysin 0
Patients with acute non-streptococcal respiratory infections	1	50	1:4	1:8
	2	<12	1:64	1:64
	3	12	1:4	1:4
	4	100	0	0
	5	50	1:128	1:128
	6	66	1:64	1:32
	7	250	0	0
	8	12	1:32	1:16
Patients with acute rheumatic fever	1	833	1:32	1:32
	2	1250	1:16	1:32
	3	625	1:4	1:4
	4	1250	Not done	1:128
	5	833	” ”	1:8
	6	625	” ”	1:8
	7	833	” ”	1:64

It was diluted 1 to 6 in saline for sensitization.

Method of performing hemagglutination tests. The modification of Smith and Scott (6), which differs only slightly from the original technic of Middlebrook and Dubos, was used in the present studies. Briefly, sheep erythrocytes were suspended in the sensitizing antigen solution for two hours at 37°C, washed gently, and resuspended in saline. Agglutination tests were performed by adding the sensitized cells to serial dilutions of heat-inactivated patients' sera. The tubes were incubated at 37°C for 2 hours, and allowed to stand overnight at room temperature. Agglutination was detected by gently shaking the tubes the morning after tests were performed. Tubes with definite macroscopic clumps were called positive; those with an abnormal cell pattern on the bottom of the tube, but without definite clumping, were called negative.

Results. Preparations A and B. Sensitization of sheep red cells did not occur with these two preparations; *i.e.*, there was no agglutination of the treated cells by sera which gave definite agglutination with preparations C and D. *Preparations C and D.* With both of these extracts there was sensitization of sheep erythrocytes with clear-cut hemagglutination in some specimens of human sera. Hemagglutination tests were performed with sera from patients with acute non-streptococcal upper respiratory infections, probably viral in etiology, and from patients with acute rheumatic fever. Representative results are presented in Table I. The hemagglutination titers were identical with the two sensitizing antigens, and there was no correlation between the antistreptolysin titers and the dilutions of sera which caused agglutination of the sensitized red cells. Finally, the hemagglutination titers recorded in patients with acute rheumatic fever fell in the same range as those observed in patients with non-streptococcal respiratory infections.

An attempt was then made to differentiate individuals with acute streptococcal sore throats from patients with acute rheumatic fever on the basis of the hemagglutination reaction. Acute and convalescent sera were obtained from 11 young adults with streptococcal sore throats. Five developed acute rheumatic fever before the second blood specimen was drawn. These sera were obtained through the courtesy of Dr. Charles H. Rammelkamp, Director of the Streptococcal Disease Laboratory, Warren Air Force Base, Wyoming. Code numbers were used for the sera, so that the results could not be influenced by knowledge of the clinical picture.

The results are presented in Table II. Definite hemagglutination was observed with all but one of the sera tested, but again there

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TABLE II. Hemagglutination Titers of Sera Obtained from 11 Young Adults with Acute Streptococcal Pharyngitis. Five developed acute rheumatic fever before the convalescent blood was drawn.

	Patient	Type of infecting streptococcus	Antistreptolysin titer (units/cc)		Hemagglutination titers	
			Acute sera	Convalescent sera	Acute sera	Convalescent sera
Patients with acute streptococcal pharyngitis who did not develop rheumatic fever	1	28	250	400	1:8	1:4
	2	14	83	250	1:16	1:32
	3	14	50	400	Not done	1:16
	4	1	62	125	1:128	1:128
	5	14	62	250	1:64	1:128
	6	14	50	317	Not done	1:16
Patients who developed acute rheumatic fever	1	14	100	317	1:16	1:16
	2	14	83	833	1:4	1:8
	3	14	125	317	1:32	1:32
	4	14	125	200	1:64	1:128
	5	14	<50	317	0	Not done

was a complete lack of correlation between the antistreptolysin and hemagglutination titers. Patients with acute rheumatic fever could not be differentiated from those with streptococcal pharyngitis on the basis of the hemagglutination test. The one consistent observation was that, for any one patient, the titers tended to be the same for both the acute and convalescent specimens.

Discussion. The present study demonstrates that various streptococcal products can be adsorbed onto sheep red blood cells, and that erythrocytes so sensitized are agglutinated by a variety of human sera. It was rather surprising to find identical hemagglutination titers with the formamide extract, which is a relatively pure preparation of the group-specific carbohydrate (C substance), and concentrated streptolysin O, which is a protein. The exact nature of the sensitizing material cannot be determined from these data, but the evidence suggests that it is the carbohydrate, with a sufficient amount of the C substance being present in the streptolysin solution to sensitize erythrocytes as effectively as the antigen in the formamide extract.

The failure of the other two preparations (A and B) to sensitize red cells is not entirely clear. The evaporated culture supernatant of intact streptococcal bodies (a preparation similar to Old Tuberculin) may simply have been less concentrated than the ammonium sulfate precipitated preparation of streptolysin O. The failure of the acid extract to sensitize cells, in contrast to the formamide extract which gave excellent adsorption, is less easily understood. It is possible that the presence of the M protein interfered in some way with adsorption of the C substance.

With human sera, clear-cut hemagglutination was present in titers as high as 1:128 with preparations C and D. There was a complete lack of correlation between hemagglutination and antistreptolysin titers, and between hemagglutination titers and the presence or absence of streptococcal infections or rheumatic fever. The one consistent finding was that the hemagglutination titers tended to be the same during the acute and convalescent stages of streptococcal infections (Table II). This suggests that the substance in the serum which is responsible for hemagglutination may have nothing to do with infection by the streptococcus, either past or present. If the serum substance is a true antibody, it may be present as a result of stimulation by an agent possessing antigenic constituents identical with certain components of the Group A streptococcus. Further study will be necessary to elucidate the exact nature of the phenomenon.

Summary and conclusions. (1) The hemagglutination technic of Middlebrook and Dubos has been applied to the study of streptococcal infections and rheumatic fever. Two of 4 streptococcal extracts gave excellent sensitization of sheep erythrocytes. These were the formamide extract, containing the group specific carbohydrate, and a concentrated solution of streptolysin O, which is a protein. Surprisingly, hemagglutination titers were identical with a variety of human sera when these two preparations were used as sensitizing antigens. (2) Hemagglutination was shown by many sera in titers ranging from 1:4 to 1:128, but there was no correlation between the magnitude of antistreptolysin and hemagglutination titers. Patients with non-streptococcal respiratory infections, streptococcal pharyngitis, and acute rheumatic fever could not be differentiated on the basis of the hemagglutination test. (3) The one consistent observation was that, for any one patient, hemagglutination titers tended to remain constant with both acute and convalescent sera. This suggests that a cross reaction of some type was responsible for the agglutination of red cells sensitized with streptococcal products. Further study is necessary to clarify the nature of this phenomenon.

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B Vitamin Content of Rat Adrenals with Respect to Exposure to Cold. (19126)

John Adams Schricker, Roy Hertz, and William W. Tullner.

*From the National Cancer Institute, Bethesda, Md.**

Sayers(5) has ably reviewed the biochemical changes observed in the adrenal gland under stress. While it has been noted that stress stimuli effect the depletion of ascorbic acid(6) and cholesterol(7) and the turnover of phosphorus(2) in the adrenal cortex, no data are available to indicate whether other substances, particularly other vitamins, are similarly affected. There is presented here the results of a study of the comparative concentration of four B-vitamins (biotin, folic acid, niacin and riboflavin) in the adrenals of rats before and after subjection to cold stress for one hour at 5°C.

Materials and methods. Female rats of the Holtzman strain, weighing approximately 150 g, were used. All had access to a stock diet of N.C.I. food pellets(3) and tap water *ad libitum,* supplemented weekly with 0.2 ml administered cod liver oil. Twenty rats constituted each experimental group. Ascorbic acid was determined by the method of Roe and Kuether(4) as described by Sayers(8) with modifications in temperature and time of incubation as outlined in the procedure below. The B-vitamins were liberated enzymatically, as recommended by Cheldelin(1), with papain and takadiastase. They were assayed microbiologically with *L. casei* in 2 ml volume, employing a medium essentially similar to that described by Tepley and Elvehjem(9) with appropriate supplements and omissions for adaptation to the several vitamin assays. The rats were anesthetized with ether, the left adrenals were removed and weighed to the nearest milligram and dropped immediately into McShan homogenizers containing one ml

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* U. S. Public Health Service, Federal Security Agency.

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TABLE I. Vitamin Content of Rat Adrenals Before and After Exposure to Cold.

Series	State	Ascorbic	Biotin	Folic	Niacin	Riboflavin
A	Before cold	3.54 ± .376	.469 ± .028	1.52 ± .129	80.4 ± 13.5	27.5 ± 3.20
	After cold*	2.20 ± .360	.465 ± .025	1.31 ± .132	68.5 ± 5.9	33.5 ± 3.33
B	Before cold	3.01 ± .470	.518 ± .039	1.29 ± .170	63.4 ± 7.6	29.7 ± 2.57
	After cold*	1.90 ± .164	.524 ± .031	1.57 ± .353	70.1 ± 7.5	31.1 ± 3.39

* Exposure for 1 hr at 5°C.

Ascorbic acid values expressed as mg per g of fresh adrenal tissue; all other values as μg per g. Each series includes 10 pre-exposure and 10 post-exposure determinations on the paired adrenal glands of 20 rats.

acetate buffer, pH 4.7. Two left adrenals, and later, 2 right adrenals, were pooled to provide a sample weight of 35-40 mg. After operation and recovery from anesthesia, each pair of rats was confined in a plastic cage placed in a cold room at 5°C for one hour. They were then killed with ether and the right adrenals taken and similarly treated.

As soon as each group of 20 was completed (*ca* 2 hours) the adrenals were homogenized and sufficient buffer added to make a 1:100 suspension. One ml of this suspension was pipetted into a test tube (19 x 150 mm) containing 9 ml trichloracetic acid for the ascorbic acid assay. The remaining suspension was transferred to 19 x 155 mm test tubes and one drop each of 2% papain and 2% takadiastase preparation added. The tubes were then stoppered and placed in an incubator at 37.5°C for 24 hours.

Ascorbic acid assay. Reagents. Trichloracetic acid, 4%; Norit A, Pfanstiehl; dinitrophenylhydrazine reagent, 2% in 9 N H_2SO_4; with 0.25% thiourea added, then filtered; sulfuric acid, 85% (9:1).

Procedure. One ml of homogenate in 9 ml of trichloracetic acid reagent was treated with 0.3 g Norit with shaking for a few moments, then filtered through a dry filter. Four ml of the filtrates were pipetted into colorimeter tubes, one ml of dinitrophenylhydrazine reagent added with mixing and the tubes were placed in a water bath at 60°C for 75 minutes. The tubes were then transferred to an ice bath and 5 ml of 85% sulfuric acid added to each, dropwise, followed by mixing. The tubes were removed from the ice bath and permitted to stand for about 20 minutes in order to come up to room temperature. They were read in a Coleman Universal Spectrophotometer at 540 millimicrons. Ascorbic acid was computed by reference to a semilog graph for standard solutions covering the working range (0-20 μg) and similarly treated.

B vitamin assay. The reagents and media have been identified above; dl-alanine was omitted in all cases and peptone was included only in the riboflavin assay. Each liter of stock media was supplemented with 20 μg of folic acid except for that used in the folic acid assay.

Procedure. Dilutions of the enzymatic digest were made to provide a 1:2000 concentration for biotin and folic acid, 1:1000 for niacin and 1:500 for riboflavin. Assays were conducted in duplicate at three levels of substrate (0.2, 0.6 and 1.0 ml) plus one ml of media. Distilled water was added to make a total of 2 ml in 13 x 100 mm tubes. The tubes were capped and sterilized at 15 lb pressure for 15 minutes. When cool, they were inoculated with one drop each of a saline suspension of thrice-washed *L. casei* cells from a 24-hour broth culture. Low blanks were assured by preparing the suspension in a dilution at least 50 times that of the original broth inoculum. After approximately 66 hours incubation at 37.5°C the cultures were titrated by 0.1 N NaOH with bromthymol blue as indicator. Vitamins were computed by reference to standard curves prepared in the usual manner.

Results and discussion. It will be seen from the data presented in Table I that the conditions of these experiments were such as to afford an extensive ascorbic acid depletion in the stressed animals. Thus the ascorbic depletion in Series A is 38% and in Series B it is 37%. In marked contrast, the changes noted in the content of biotin, folic acid, niacin, and riboflavin are very limited and within the expected variability of the microbiological methods employed. These observations lend increased specificity to the ascorbic acid depletion effect since they indicate that other water soluble factors, which are also intimately involved in cellular metabolism, are not as readily altered by stress.

The concentration of ascorbic acid in the adrenal is very high in comparison with other tissues of the body(4). The observed adrenal concentrations of biotin, folic acid, niacin and riboflavin are of about the same order as previously found for rat liver and kidney(1). This contrast also serves to emphasize the special significance of the high adrenal content of ascorbic acid.

Summary. In contrast with the expected extensive depletion of ascorbic acid, the biotin, folic acid, niacin and riboflavin content of the rat adrenal gland was observed not to be materially altered by exposure to cold.

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Effect of Starvation on Exchangeable Potassium and Tissue K^{42} Content in Rabbits.*† (19127)

JERRY K. AIKAWA.‡ (Introduced by G. T. Harrell.)

From the Department of Internal Medicine, Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, N. C.

In the course of a clinical survey of individuals with various diseased states, low values for exchangeable potassium, as measured by the radioactive isotopic technic, were encountered(1). In general, these low values appeared to be due to: (1) a decrease in body mass secondary to increased catabolism produced by the disease process; (2) a diminished intake of food from anorexia induced by the illness; (3) an intracellular deficiency of this cation from some inherent metabolic abnormality; or to a combination of these factors. The role of human disease and of metabolic abnormalities would be best studied in patients who present such problems. The effect on potassium metabolism of a decrease in food intake in the absence of disease can be approached experimentally. Since such a study was not feasible on human beings in our laboratory, rabbits were starved and the changes produced were followed serially.

The purpose of the present study is to determine the effect of starvation for a short period of time on the total potassium content of the body, as well as its concentration in various tissues. Because of the greater correlation of weight and urinary creatinine with exchangeable potassium content in human males as compared with females, the following experiments were performed in male animals (2,3).

Material. Nineteen normal male adult rabbits of mixed breeds were used. They were placed in individual metabolism cages and were fed a stock diet of compressed pellets. Tap water was given without restriction.

Methods. Experimental procedure. The experiment was planned to study the alterations in potassium metabolism which would be produced by a reduction in body weight of ap-

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† The technical assistance of Miss Eloise L. Rhoades and Mr. Claude Taylor is gratefully acknowledged.

‡ Research Fellow of the American Heart Assn.

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proximately 15%. A loss in weight of this extent is not uncommonly encountered clinically and would be comparable to a decrease of 12.5 kg (22.5 lb) in a 70 kg (150 lb) man. Because of the technical difficulties involved in the use of an isotope with such a short half-life (12.4 hours), an interval of one week was found to be convenient between isotopic determinations. Group A consisted of 7 animals which were observed for a control period of one week before starvation and subsequently during the deprivation of food. External potassium balance studies were conducted for the baseline period and the first week of starvation. At the conclusion of this period the animals were still in good condition, so that the observations with radiopotassium were continued for an additional week, but balance studies were not done. Each morning before feeding, the body weight, total food consumption and the daily urine volume were recorded. Creatinine determinations were performed in the morning on an aliquot of 24 hour daily sample. Baseline exchangeable potassium determinations were completed in the morning just before food was withdrawn. This determination was repeated after 1 and 2 weeks of starvation. Six rabbits (Group B) were fed for a period of one week to establish equilibrium on the diet. Food was then withdrawn completely, though water was allowed *ad libitum*. After one week the animals were sacrificed by exsanguination and analyses for radiopotassium and total water content were performed on 17 different tissues. Another group of 6 animals (Group C) served as a control for Group B. These rabbits were continued on food and water *ad libitum* but otherwise were treated identically with Group B.

Radioisotope methods. The preparation of the potassium solution for injection has been previously described(2). A calibrated volume of 5 ml of the K^{39} solution containing 30 μc of K^{42} in 0.5 milliequivalent was injected intravenously. The urine was collected for 24 hours; a spot specimen was then obtained by catheterization and its specific activity was determined. The following formula was used to calculate the value for the *exchangeable potassium content* of the body:

$$Ke = \frac{K_i{}^{42} - K_o{}^{42}}{\frac{Ku^{42}}{Ku^{39}}}.$$

K_e = quantity of exchangeable K in milliequivalents.

$K_i{}^{42}$ = quantity of radiopotassium administered (arbitrary units).

$K_o{}^{42}$ = quantity of radiopotassium excreted in urine until spot specimen was obtained.

Ku^{42}/Ku^{39} = specific activity of spot specimen.

Tissue analyses. Three aliquots (1 to 3 g) of each tissue were obtained. They were kept in covered Petri dishes and were weighed as rapidly as possible on a Gramatic balance to 0.1 mg. Two portions were digested in concentrated sulfuric acid for determination of *radiopotassium concentration.* The digest was made up to a volume of 20 ml and counted with a dipping tube and a scaling circuit. The tissue concentration of radiopotassium was expressed as per cent of the injected dose per g of wet tissue. *Tissue water content* was determined on the third aliquot, which was dried to constant weight at 110°C for 18-24 hours. The *potassium* concentration in the urine was determined by flame spectrophotometry by a method which has been previously described (4). Urinary *creatinine* concentration was determined by a modification of the method of Bonsnes and Taussky(5). *External balance of potassium.* Each g of feed was found to contain 0.159 meq. of potassium. The tap water contained a negligible amount. The potassium content of the ingested feed was found to be completely absorbed through the gastrointestinal tract. The content of potassium in the stool was too low to be detected by flame photometry. The loss of potassium from the body was, therefore, calculated on the basis of its urinary excretion. *Statistical method.* The significance of the difference between the means was derived by the use of the "t" test(6).

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Results. The 7 animals followed by external balance and K_e determinations (Group A) were in potassium balance and gained weight during the baseline period (Table I, Fig. 1). During the period of starvation a progressive loss of body weight occurred. The mean weight loss for the group after the first week was 0.35 kg (15.6% of the mean initial weight) (Table I, Fig. 1); this amount, however, was not statistically significant. After 2 weeks, the mean decrease in weight amounted to 0.71 kg, which is significant. At the end of the first week the mean negative potassium balance amounted to 23 meq. The mean K_e decreased 41 meq. during the first week and an additional 34 meq. during the second week of starvation. By the end of the second week of starvation, a significant reduction in K_e/wt had occurred.

Although the mean creatinine excretion decreased 146 mg during the first week and 124 mg during the second week, as compared with the baseline values, these changes, as well as the decreases in the creatinine coefficient, were not significant. The urine volume increased the first week of starvation.

Discussion. The present results emphasize the importance of the concept that the body potassium is in dynamic equilibrium, and that the changes in the rate of exchange may be the first indication of a disturbance in its metabolism. Such an abnormality may occur before any alteration in concentration of potassium in blood or tissue is detectable.

During starvation, as tissue is catabolized, the weight decreases progressively and the total body content of exchangeable potassium decreases. The continued loss of potassium in the urine during starvation confirms the endogenous source of this cation. With tissue catabolism, the K_e to weight ratio decreases at a relatively constant rate, at least for the first two weeks of starvation.

The exchangeable potassium loss after a week of starvation exceeds by 18 meq. the potassium loss as measured by the external urinary balance method. According to the method of calculation of K_e, the more in-

TABLE I. Effects of Starvation on Potassium and Creatinine Metabolism in Normal Male Rabbits (Group A).

Starvation	Wt, kg	Exchangeable K (meq)	Ke/wt (mg/kg)	Urinary creatinine (mg/wk)	Creatinine coef. (mg/kg)	Urine vol (ml/wk)	Oral intake K (meq/wk)	Urinary K^{39} excretion (meq/wk)
Before	2.24 ± .33*	137.4 ± 15.4	62 ± 5.43	694 ± 207	45.8 ± 4.85	901 ± 356	77.8	76.6
1st wk	1.89 ± .33	96.4 ± 23	51.8 ± 14.80	548 ± 155	38.9 ± 7.95	1456 ± 165	0	23
2nd wk	1.53 ± .35	62.4 ± 11.7	41.4 ± 4.45	424 ± 160	34.8 ± 7.92	1149 ± 355	0	—

* Standard deviation.

Results of Balance Studies

Starvation	wt	Exchangeable K (meq)	Ke/wt (meq/kg)	Urinary creatinine (mg/wk)	Creatinine coef. (mg/kg)	Urine vol (ml/wk)	Urinary K^{39} excretion (meq/wk)
1st wk	—.35	—41*	—10.2	—146	— 6.9	+555*	+23
2nd wk	—.71*	—75*	—20.6*	—270	—11	+248	—

* Statistically significant difference when compared with baseline values. $P = < .01$.

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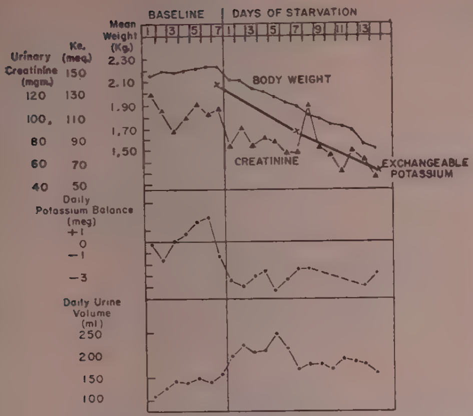

FIG. 1. The effect of starvation on potassium and creatinine metabolism in normal male rabbits.

complete the exchange of radioactive atoms with the normal intracellular untagged potassium ions, the smaller would be the calculated exchangeable potassium content. In normal rabbits approximately 90% of the body potassium would have exchanged in 24 hours(2). It appears that with starvation and the consequent reduction in metabolic rate, which is known to set in quickly during fasting, the rate of turnover of intracellular potassium is decreased, or the relative amount of "bound" potassium is increased.

Comparison of the values in the normal animals (Group C) and the rabbits starved for one week (Group B) revealed no significant difference in the concentrations of tissue water or tissue radiopotassium for the following tissues: skin, subcutaneous connective tissue, abdominal muscle, gastrocnemius muscle, quadriceps muscle, diaphragm, tendon, cartilage, heart ventricle, heart auricle, lung, liver, spleen, stomach, appendix, adrenal and kidney. The mean weight loss of the starved animals after one week of starvation was 0.43 kg (13.8% of the initial mean body weight). Since the tissue analyses show no significant difference, it is possible, therefore, to account for the total decrease in body potassium on the basis of tissue breakdown without postulating an intracellular deficiency in the remaining tissues.

The creatinine coefficient serves as an index of relative muscle mass. Although the decrease in this ratio observed with starvation may imply 1) a decrease in the rate of metabolism of muscle tissue; 2) a decrease in the total muscle mass, or both, the differences at one and 2 weeks were not significant when compared with the baseline value. With diminished intake of food, a relatively greater utilization of tissue fat and carbohydrate in preference to protein would be expected initially. The utilization of fat was evident grossly at the time the samples for analyses were obtained; the amount of perirenal fat in the starved animals, for instance, was considerably less than in the control rabbits, though the muscles appeared similar.

Since no significant change in radiopotassium concentration was found in any specific tissue or organ in starved animals, the decrease in rate probably was generalized over the body. The possibility that such an alteration might be limited to the skeletal or nervous system must be considered, since these two tissues were not included in the present analyses. The radioactive potassium method, therefore, appears to be a more sensitive index of the functional defect produced by starvation than the external balance method.

The possibility that the loss of potassium in the feces during starvation was increased over the normal amount must also be considered. In such an event, the urinary balance study alone would underestimate the total loss. However, since, during the prestarvation period, there was no loss in the stool, it would be highly unlikely that the discrepancy of 18 meq. between the external urinary balance and the K_e could be explained on this basis alone.

These studies complement our clinical observations and lend support to the hypothesis that whenever catabolic processes exceed the anabolic, the loss of body potassium, weight, and muscle tissue parallel each other(1).

Summary. 1. Male rabbits were starved in order to study the effect of a decrease in food intake on potassium metabolism. 2. Serial determinations of the exchangeable potassium content and urinary potassium and creatinine excretion were made in one group of animals. The mean decrease in exchangeable potassium

content was 29.8 and 54.6% of the baseline value after one and two weeks respectively. The corresponding mean decrease was 15.6 and 31.7% in body weight. No significant changes in urinary creatinine excretion occurred. 3. In a separate group of rabbits, tissue analyses for radiopotassium and water concentration after one week of starvation revealed no significant changes when compared with control animals, continued on a constant diet. 4. The decrease in total body potassium content, as measured by the exchange of isotopic potassium, as well as the loss in the urine during starvation could be accounted for on the basis of tissue catabolism without postulating an intracellular deficiency of this cation in the remaining tissues. A functional abnormality in potassium metabolism may be detectable by the radioisotope method prior to its manifestation by the external balance method.

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Cholic Acid: An Adequate Stimulus for Hypercholesteremia in the Normal Fasting Rat.* (19128)

MEYER FRIEDMAN AND SANFORD O. BYERS.

From the Harold Brunn Institute for Cardiovascular Research, Mount Zion Hospital, San Francisco, Calif.

Recent studies(1,2) in this laboratory have indicated that the administration of sodium cholate to a rat with biliary obstruction markedly increases the degree of hypercholesteremia ordinarily occurring after obstruction. This effect of cholate could be obtained as well in the starved as in the fed animal. Moreover, parenteral injection of cholate was as effective as oral administration of the drug.

These facts suggested to us the possibility that perhaps intravenous administration of cholate to the normal, intact, starved rat might in itself produce hypercholesteremia. The present report indicates the results of such administration of cholate.

Methods. Male rats (Long Evans) approximately 10 weeks of age, were operated upon under ether anesthesia. One end of a flexible polyethylene cannula (diameter: 0.011 inch) was inserted into the inferior vena cava of each animal via a lumbar tributary. The other end of the cannula was attached to a syringe whose piston was gear driven in a special apparatus† at such a rate as to deliver 0.3 ml of fluid per hour. The abdominal incision was then closed and the rats were placed in individual Bollman cages(3). In this manner, a preparation was obtained which allowed the *continuous* intravenous injection of fluid into unanesthetized rats. Six of the animals were given sodium cholate in Tyrode's solution at a rate equivalent to 25 mg of cholic acid per hour. The remaining eight rats received Tyrode's solution, alone, thus serving as controls. Each of the experimental rats received approximately 500 mg of cholate per day. The rats were starved for 24 hours before operation and during the experiment. Blood samples taken before and 24 hours after the beginning of the injection were analyzed for bile acids and cholesterol in plasma according to previously described methods(4,5).

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* Aided by grants from the American Heart Association and The U. S. Public Health Service.

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TABLE I. Effect of Intravenous Cholate on Plasma Cholesterol of Normal Rat.

Wt, g	Amt cholic acid given (mg/24 hr)	Cholesterol (mg/100 cc)		Bile acid (mg/100 cc)	
		Before inj.	24 hr after inj.	Before inj.	24 hr after inj.
		(Rats given cholate)			
320	570	57	133	6.3	17.8
226	500	70	140	6	29
202	500	73	107	3	18
207	500	54	140	1	—
279	503	46	91	3	23
220	500	48	96	5.4	32
Avg 241		58	118	4.1	24
		(Control rats)			
191	—	56	60	8	7
168	—	58	88	2	5
211	—	82	55	1	3
238	—	71	74	4	4
192	—	64	85	4	4
200	—	48	56	—	—
210	—	41	53	—	—
205	—	40	49	—	—
Avg 201		58	65	3.8	4.6

Results. As Table I indicates, experimental elevation of the plasma bile acid was followed by elevation of the plasma cholesterol. The average content of bile acid increased from 4.1 mg to 24.0 mg per 100 cc during the injection period. The average plasma cholesterol increased from 55 mg to 118 mg per 100 cc. No significant changes occurred in the cholesterol content of the control rats which had not received cholate.

Discussion. The rats given cholate became hypercholesteremic despite the fact that they were *starved* and the substance was given *parenterally*. These facts make it clear that the cholate effect could not have been due to some theoretical alteration in the intestinal absorption of steroids. Moreover, we found in a previous study(2) that cholate alone, of 4 different bile acids examined, exerted this hypercholesteremic effect in the rat with biliary obstruction. It seems, therefore, most likely that the action of cholate is a specific one.

The experimental production of hypercholesteremia by *prior* injection of cholate is important because it suggests that hypercholesteremic conditions, if accompanied by an excess of plasma cholate, may have their origin in the accumulation of plasma cholate. In other words, various hypercholesteremic states heretofore considered primary or idiopathic may be instead, secondary to an *initial* derangement of cholate metabolism. Certainly it seems important to re-investigate hypercholesteremic conditions with this possibility in mind.

Summary. Experimental hypercholesteremia was produced by intravenous injection of sodium cholate into normal fasting rats. The implications of this finding were discussed.

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Excretion and Distribution of Radioactive S^{35}-Cyclamate Sodium (Sucaryl Sodium) in Animals.*† (19129)

J. D. Taylor, R. K. Richards, and Jeanne C. Davin.

From Abbott Laboratories, North Chicago, Ill.

Sodium N-cyclohexylsulfamate (sucaryl sodium) has been introduced recently as a non-caloric sweetening agent. It was shown previously that cyclamate sodium‡ was excreted unchanged to the extent of 80-90% in 12 hours in the urine of rabbits when given intravenously, intraperitoneally, or orally(1). In man given 0.3 g of sucaryl in a preliminary experiment(2) 26% of the dose was excreted the first 24 hours and 79.5% excreted after 7 days. The methods used were insensitive and required relatively large doses. In an attempt to overcome these limitations and to extend our work in animals, sucaryl sodium containing radioactive S^{35} was synthesized.

This paper is concerned with the excretion and distribution of S^{35}-sucaryl§ in animals.

Experimental. Young, adult, white rats were the principal animals studied; their urine and feces were collected separately. In one experiment a male dog was anesthetized with pentobarbital and the carotid artery, bile duct, and the ureters were cannulated. After intravenous injection of S^{35}-sucaryl sodium, samples of bile, blood, and urine were taken at various time intervals up to 5 hours, at which time the dog was killed and samples of tissue were taken for analysis. The analytical method used for the estimation of S^{35}-sucaryl was a modification of that previously described(1,2) and adapted for determination of S^{35} in biological materials. In all of these experiments S^{35}-sucaryl sodium originally containing 7.06 millicuries per g (2.2 x 10^6 counts/minute/mg) was used through 3 half-lives. One to 4 g of tissue were weighed into a 300 ml Kjeldahl flask and 10 ml of concentrated HNO_3 were added and washed down with 50 ml of water. The contents of the flask were boiled, using a microburner, and slowly evaporated until brown fumes appeared. After cooling, distilled water was added and the solution was filtered through Whatman No. 42 filter paper, washing well with distilled water. The filtrate was made to about 500 ml with distilled water, neutralized to pH 6-8 with 25% NaOH and carrier sulfate added in the form of sulfuric acid or Na_2SO_4 equivalent to about 100 mg of $BaSO_4$. Ten ml of concentrated HCl were added, stirred, and then 10 ml of 10% $BaCl_2$ were added slowly and the mixture allowed to stand 1 hour without stirring. The sample was stirred and heated on the steam bath to digest. The samples were cooled to room temperature and filtered through tared filtering crucibles (Selas No. 3010). The precipitate was washed well with water, then alcohol, and finally dried in an oven at 130°C. After cooling to room temperature, the crucibles were weighed.

Ten to 30 mg of the radioactive $BaS^{35}O_4$ were removed from the crucible and plated on a 1-inch tared Cu disk. The $BaS^{35}O_4$ was spread evenly with the aid of a drop or two of water over a fixed inscribed area of 3.56 cm^2 and dried under a heat lamp. The disc was weighed when cool. From these data the mg/cm^2 was calculated. The disc was counted under an end-window Geiger counter (1.8 mg/cm^2 window). Counts per minute were corrected for decay and for self-absorption using an empirically determined calibration curve. From this value the sucaryl content was calculated, assuming that all the radioactivity was unchanged S^{35}-sucaryl sodium. An assay of the original solution used was performed simultaneously and all values were referred to it. On replicate determination of S^{35}-sucaryl added to urine, feces, and tissues, recoveries of 95±5% (av. dev.) were obtained. An alternative method used on urine was to pipette an aliquot of the suitably diluted urine onto a tared Cu disc. After drying, weighing, and counting, the counts/min. were corrected by referring to an empirically determined curve. This gave values that agreed within 10% of those obtained with the $BaSO_4$ method.

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* Sucaryl sodium is the registered trademark of Abbott Laboratories, North Chicago, Ill.

† We are grateful to G. I. Gleason and D. L. Tabern, Department of Radioactive Pharmaceuticals, for the synthesis of this compound.

‡ Cyclamate sodium has been accepted as the generic name for sucaryl sodium.

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§ In this paper S^{35}-sucaryl sodium will be referred to in some cases as S^{35}-sucaryl because only the anionic portion of the molecule is radioactive and measurable. However, all values are calculated on per cent of dose of sucaryl sodium.

Results and discussion. Preliminary experiments showed that there was no demonstrable exchange between the S^{35} of sucaryl sodium and Na_2SO_4 *in vitro* at room temperature in 24 hours. Experiments on the urine of rats fed S^{35}-sucaryl sodium using radiopaper chromatographic methods showed that less than 0.15% of the 100 mg kg dose was $S^{35}O_4$ ion. The remainder of the radioactivity was concentrated in a single band having an Rf value comparable to that of pure S^{35}-sucaryl sodium. Cold isotope dilution experiments on rat urine fed S^{35}-sucaryl indicated that at least 95% of the sucaryl was excreted unchanged. The possibility of aromatization of the cyclohexane ring(3,4) was not investigated, but from the isotope dilution experiments and the paperchromatograms it is assumed in this work that sucaryl was excreted unchanged and that the measurement of S^{35} content of any tissue or fluid is also a direct measurement of the unchanged drug.

Excretion. The data on the excretion of S^{35}-sucaryl by the rat given a single dose intraperitoneally is shown in Table I. With a dose of 90-410 mg/kg an average of 60% was found in the urine in 3 hours and 72% in 5 hours. In 1 animal given an intravenous injection of 83 mg/kg, 75 and 77% of the dose was excreted by the kidney in 3 and 5 hours respectively.

TABLE I. Excretion of S^{35}-Sucaryl in Urine of the Rat Injected Intraperitoneally. Animals injected intraperitoneally with 10 ml of .9% NaCl at time of dosing.

Dose, mg/kg	No. of rats	% of original dose excreted in the urine 2 hr	3 hr	4 hr	5 hr
90	1	36	51	55	56
105	1	68	80	83	84
110	1	—	62	85	86
410	1	56	67	67	72
410	1	—	40	40	62
Avg			60	66	72

TABLE II. Excretion of S^{35}-Sucaryl by the Rat Given a Single Dose. Excreta from two rats pooled for analysis.

Route admin.	Dose, mg/kg	% of original dose excreted in urine and feces 1 day	2 days	4 days	Distribution of excreted S^{35}, % Urine	Feces
Oral	100	75		101	54	47
”	100	58		99	67	33
Subcut.	100	92		94	93	7
I.P.	650	94	106		79	21
”	1250	71	78		76	24
”	2450	82	91		87	13
”	3200	81	86		90	10

Table II shows the excretion of S^{35}-sucaryl over longer periods of time. At the end of 4 days, an average of 98% of the radioactivity was recovered in the urine and feces in 3 experiments on 6 rats. Increasing the intraperitoneal dose about 4 times only slightly lowered the amount excreted the first day. At the largest dose of 3.2 g/kg, 81% of the radioactivity was excreted in 24 hours. In one experiment one kidney was removed and 24 hours later a dose of 1.4 g/kg was administered intraperitoneally. At the end of 24 hours, 70% of the dose was excreted. This amount is not significantly different from the amounts excreted by a rat with both kidneys intact and given a comparable dose. The relative proportion of a single dose of sucaryl excreted by the kidney and the intestines varied depending on the route of administration and the amount of the drug given. Of the total amount excreted about 40% of an oral dose of 100 mg/kg was excreted in the feces in 4 days. Subcutaneous injection of the same dose resulted in a decrease in the

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TABLE III. Excretion of S^{35}-Sucaryl by the Rat Given 5 Repeated Doses. Excreta from two rats pooled for analysis.

Route admin.	Dose, mg/kg/day	% of dose excreted in urine and feces: 5 days	% of dose excreted in urine and feces: 9 days	Distribution of excreted S^{35}, %: Urine	Distribution of excreted S^{35}, %: Feces
Oral	120	95	100	37	63
”	120	78	81	33	67
”	80	81	82	32	68
Avg		84.7	87.7	34	66
I.P.	140	93	94	98	2

TABLE IV. S^{35}-Sucaryl Content of Blood, Bile, and Urine at Various Times in the Dog Injected Intravenously with 9.7 mg/kg Sucaryl.

Time after inj., hr	Blood, mg/ml	Bile: mg/ml	Bile: % dose inj.	Urine: mg/ml	Urine: % dose inj.
.5	13.6	—	—	—	—
1	—	35	.2	4	34.4
1.5	5.9	—	—	—	—
2	—	34	.2	2	17
3	2.6	24	.07	.8	7.6
4	2	16	.03	.4	4
5	.9	8	.02	.2	3.8
Total			.52		66.8

proportion excreted in the feces to 7%. Increasing the dose injected intraperitoneally resulted in a decreased proportionate amount excreted by the intestines over a period of 2 days.

The data in Table II showed that a single dose of sucaryl sodium was almost completely excreted by the rat. Table III summarizes the data on rats given repeated oral doses for 5 consecutive days. An average of 85% was excreted in 5 days and another 3% in the 4 days following the last dose. On intraperitoneal injection, 93% was excreted in 5 days with only 1% eliminated in the 4 days after the last dose. These low recoveries of excreted sucaryl 4 days after the last dose are probably due to accumulated losses during these experiments, as the carcasses of these animals yielded less than 1% of the total dose at this time. About two-thirds of the oral dose was excreted in the feces, the remainder in the urine. On repeated intraperitoneal injection only 2% of the dose was found in the feces. In one experiment rats were fed a diet containing 1% of cold sucaryl sodium for 54 days, following which a single dose of 100 mg/kg of S^{35}-sucaryl sodium was injected intraperitoneally. An average of 77% of this dose was excreted in 24 hours, indicating that feeding of sucaryl sodium for this period of time did not significantly influence its excretion. From these data it was concluded that sucaryl was not stored in the body of the rats to any significant extent.

Table IV shows the data on a dog injected intravenously with 9.7 mg/kg of S^{35}-sucaryl and with the bile duct and ureters cannulated. Only 0.5% of the total dose was excreted into the bile at the end of 5 hours as compared to 66.8% in the urine in the same time. Evidently the bile is not an important route of excretion in the dog. It is probable that the large amounts of sucaryl excreted by the rat in the feces is partially the result of incomplete absorption from the lumen of the intestine and not of re-excretion by way of the bile.

Tissue distribution. Table V summarizes the data on the distribution of sucaryl in the rat, rabbit, and dog. Generally, the animals did not concentrate sucaryl in any one tissue to any significant extent except the kidney, and less regularly in the liver. There was considerable variation from rat to rat 4 hours after injection. This was probably caused by differences in the rate of excretion from animal to animal. At 2.5 hours there was great diversity in concentration of sucaryl among the various tissues; at 24 hours the tissue contents were similar in concentration. This was probably caused by the relatively long time necessary for the equilibrium to develop between blood and the individual tissues. There is practically no sucaryl in brain or cerebrospinal fluid during the first few hours after administration, indicating that the blood-brain barrier is relatively impermeable to sucaryl. From the large amount of sucaryl found in the fetus it is evident that it penetrates the placental membrane. The data also indicate that sucaryl is not evenly distributed throughout the body water, at least during the first 4 hours following injection.

Summary. 1. The excretion and distribution of S^{35}-sucaryl in the tissues of the rat, dog, and rabbit were studied. 2. By means

TABLE V. Distribution of Intravenously Injected S^{35}-Sucaryl in the Tissues of Animals. Values in mg/g of tissue (wet wt).

Species	Rat					Rabbit	Dog
Dose	100 mg/kg					50 mg/kg	9.7 mg/kg
Time, hr	2.5	4	4	4	24	1	5
Tissue							
Blood	104	.18	.37	.7	.13	12.4	.92
Brain	1.5	.05	.05	—	.11	.6	.07
Kidney	50	1.4	3.13	3.5	.34	72	2.77
Liver	159	1.3	.38	—	.10	4.3	1.89
Heart	25	.82	.46	—	.10	—	.86
Lungs	17	.31	.43	—	.07	—	.94
Spleen	7	.30	.41	—	.29	—	.79
Intestine	9	2.70	.31	—	.31	—	.83
Skin	9	—	.25	—	.05	—	.54
Muscle	5	.20	.10	—	.22	11.5	.41
Bone	.3	1.35	.27	—	.37	—	1.11
Testicles	—	.80	—	—	.08	—	.97
Eyeballs	.3	—	—	—	—	—	—
Adrenals	.1	.52	.00	—	.00	—	—
Pancreas	—	—	—	—	—	—	.76
Cerebrospinal fluid	—	—	—	—	—	.5	—
Fetus	—	—	—	3.5	—	—	—

of cold isotope dilution and paperchromatographic methods, it was shown that in the rat at least 95% of the material was excreted unchanged. 3. Single doses up to 3.2 g/kg intraperitoneally were rapidly excreted by the rat in 24 hours. A dose of 1.4 g/kg was excreted with equal rapidity by normal and a unilaterally nephrectomized rat. 4. With repeated oral dosing in rats about two-thirds of the drug was excreted in the feces and one-third in the urine. No significant amounts of S^{35}-sucaryl were found to be stored in the body. 5. In the dog only 0.5% of the injected S^{35}-sucaryl was found in the bile 5 hours after an intravenous injection of 9.7 mg/kg. 6. With the exception of the kidney and perhaps the liver, there was no significant concentration of the drug in the various organs of the rat, rabbit, and the dog. Sucaryl penetrated into the brain with difficulty, but was found in the fetus of the rat.

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Serum Lipase (Tributyrinase) in Hypertension and Arteriosclerosis.*
(19130)

ADOLPH BERNHARD. (With the technical assistance of Ann Rothenberg.)
From the Achelis Laboratory, Lenox Hill Hospital, New York City.

Changes in serum lipase activity have been observed in patients with disturbed pancreatic function and with carcinoma of the gastrointestinal tract(1-4). As far as we can ascertain, no detailed studies have been made of the lipolytic activity of the serum in persons with hypertension or arteriosclerosis. This paper records such a study in normal, hypertensive, and arteriosclerotic individuals.

Methods and materials. The technic em-

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TABLE I. Statistical Analysis of Serum Lipase (Tributyrinase) Activity of Human Serum.

Group	No. of cases	Range per 100 ml serum	Mean	Stand. dev.
		Male		
Normal	25	120-200	160.8	21.5
Hypertensive	28	70-185	133.8	26.1
Arteriosclerotic	31	90-195	137.4	28.7
		Female		
Normal	26	90-155	127.5	22.9
Hypertensive	15	90-205	139.6	33.6
Arteriosclerotic	25	85-155	120.9	19.7

ployed using aqueous tributyrin as the substrate, was that described by Goldstein, Epstein, and Roe(5), with the following modifications; a Waring Blender was used in the preparation of the emulsion, and the control tube was inactivated for ½ hour at 57°C. A tributyrinase unit is the amount of enzyme that will catalyze the hydrolysis of tributyrin with the release of 1 ml of 0.1 N fatty acid in one hour at 37°C, with an end point at pH 10.65. All results are given in terms of tributyrinase units per 100 ml of serum. In order to evaluate the method, determinations of serum tributyrinase were made on the same individual daily over a period of one week. The results were consistent and within the error of the method.

Results. Table I records the results of our studies of serum tributyrinase activity in normal, in hypertensive and in arteriosclerotic males and females. The normals were healthy laboratory workers, nurses, and internes. The age of this group ranged between 20 and 40 years for the males, and 19 to 35 for the females. The blood pressures varied between 90/50, and 130/80 mm of mercury. In normal females the serum tributyrinase concentration is definitely lower than that found in males, the age distribution being approximately the same. This observation confirms the work of other investigators(5,6).

Hypertension. The criterion used for determining hypertension was a systolic pressure of 150 mm of mercury or over with a diastolic pressure of 90 mm of mercury or over(7). In 28 hypertensive males between the ages of 44 and 83 years, the tributyrinase activity was lower than that of the normotensive male individuals, while the values obtained in hypertensive females with the same age distribution showed no difference from the normal. When compared with the normals, the p value of the male hypertensive was statistically significant, while that of the female hypertensive group showed no difference(8). There was no definite relationship between the serum tributyrinase activity and the age or blood pressure of the patients studied.

Arteriosclerosis. In 31 males between the age of 39 and 83 years showing clinical evidence of arteriosclerosis, but without an increase in blood pressure, the serum tributyrinase concentration was lower than that of the male normotensive group. Again the p value was statistically significant when compared with the normals. The serum tributyrinase concentration in female arteriosclerotic patients showed no difference from the female normal group.

Discussion. The results obtained seem to indicate that there are increases in the serum tributyrinase activity of hypertensive and arteriosclerotic males and not in the females. The p values of both groups of males when compared with the normals are statistically significant, while those of the females are not.

A comparison of the means of the female hypertensive and arteriosclerotic groups with that of the normal showed no difference, however, a significant statistical difference was observed, when the means of the hypertensive and arteriosclerotic groups are compared. A similar comparison of the means of the male hypertensive and arteriosclerotic groups did not reveal such differences.

Summary and conclusions. A study of the tributyrinase activity of the serum of normotensive, hypertensive, and arteriosclerotic male and female adults is presented. There

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was a decrease in the tributyrinase of the serum of hypertensive and arteriosclerotic males as compared with the normal, while the serum of the females shows no difference from the normal.

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Growth-Promoting Activity of L-Lyxoflavin.*† (19131)

H. W. BRUINS, M. L. SUNDE, W. W. CRAVENS, AND E. E. SNELL.‡

From the Departments of Poultry Husbandry and Biochemistry, University of Wisconsin, Madison.

Pallares and Garza(1) isolated L-lyxoflavin from the heart myocardium. These workers did not suggest any biological significance of this finding, but recently Emerson and Folkers(2) found that lyxoflavin stimulated the growth of rats on a hyperthyroid soybean ration, equal to the response given by an insoluble liver fraction.

A chick assay for unidentified factors has been described by Bruins *et al.*(3). Briefly, the diet is an isolated soybean protein-sucrose diet, properly supplemented with salts, known vitamins and aureomycin. Thiamine is supplied by injection. It seemed desirable to test the activity of L-lyxoflavin under these conditions; accordingly studies were initiated in April, 1951.

Results and Discussion. A summary of the results of 5 experiments with chicks is given in Table I. These results show that lyxoflavin consistently elicits a growth response, alone and in combination with dried whey, wheat bran and liver residue. The magnitude of this response is not large, being of the order of a 10% increase. Certainly, it does not represent all of the unidentified factor activity involved.

In fact, these results do not clearly show what crude supplements might owe their activity to their content of lyxoflavin. As a working hypothesis, it was earlier believed that whey, being a good source of riboflavin, might fall into this class, but that has not been demonstrated in these trials since lyxoflavin complements a level of whey which is believed to be sufficient to supply the "whey factor"(4).

The mode of action of this compound is not elucidated by these trials. It will be noted in Experiment 5 that lyxoflavin does not have riboflaviin activity for the chick, even though these compounds differ only in the configuration about C_4 of the side chain. Emerson and Folkers reported similar results for the rat.

Snell *et al.*(5) showed that lyxoflavin similarly is without riboflavin activity for microorganisms, although its addition in appropriate quantities does stimulate growth of *Lactobacillus casei* when riboflavin is limiting. At high concentrations, lyxoflavin inhibits growth of this microorganism, acting as an antimetabolite of riboflavin.

The possibility exists that the growth-promoting action of lyxoflavin for chicks might be due to a similar "antibiotic" activity. However, no action of this sort is indicated since the basal ration contains 50 mg/kg of aureomycin, a level more than adequate to promote maximum antibiotic response. That combinations of antibiotics might stimulate growth

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† We are indebted to Merck & Co., Rahway, N. J., for supplying the lyxoflavin and to Lederle Laboratories, Pearl River, N. Y., for the aureomycin.

‡ Now with the University of Texas, Austin, Texas.

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TABLE I. Tests with L-lyxoflavin.

Exp. No.	Chicks per group*	LSD, g	Group No.	Additions to basal ration†	Two wk wt, g‡
1	10	—	1	0	90
			2	3 mg/kg L-lyxoflavin	114
			3	5% wheat bran	115
			4	5% wheat bran + 3 mg/kg L-lyxoflavin	132
			5	10% dried whey + 10% liver residue	168
2	14	13.7	1	0	85
			2	3 mg/kg L-lyxoflavin	84
			3	15 mg/kg L-lyxoflavin	109
			4	5% wheat bran	110
			5	5% wheat bran + 3 mg/kg L-lyxoflavin	128
			6	5% wheat bran + 15 mg/kg L-lyxoflavin	129
3	13	9.8	1	0	87
			2	5 mg/kg L-lyxoflavin	93
			3	10 mg/kg L-lyxoflavin	97
			4	15 mg/kg L-lyxoflavin	96
			5	30 mg/kg L-lyxoflavin	99
			6	4% dried whey + 4% liver residue	153
4	13	14.8	1	0	83
			2	2% liver residue	99
			3	2% liver residue + 15 mg/kg L-lyxoflavin	108
			4	2% liver residue + 4% dried whey	119
			5	4% liver residue + 4% dried whey	128
5	12	14.8	1	0	94
			2	15 mg/kg L-lyxoflavin	111
			3	4% liver residue	132
			4	4% liver residue + 15 mg/kg L-lyxoflavin	145
			5	4% dried whey	115
			6	4% dried whey + 15 mg/kg L-lyxoflavin	125
			7	4% dried whey + 4% liver residue	151
			8	Less riboflavin	51
			9	Less riboflavin + 15 mg/kg L-lyxoflavin	54

* Chicks weighed wing banded and randomized among groups and placed on experimental rations at day old.

† Supplements were made at the expense of sucrose.

‡ Mortality was negligible in all groups.

beyond what would be observed from a single antibiotic has been suggested, but thus far no evidence in support of this suggestion has been published.

In preliminary experiments, the supplementation of simplified practical-type rations with lyxoflavin has resulted in slight growth stimulation. However, further experiments are under way to evaluate the practical significance of this result.

Summary. L-lyxoflavin stimulates growth of chicks under these conditions. Its mode of action remains obscure. Since it has no riboflavin activity, and its growth-promoting action occurs on a ration rich in riboflavin, it is quite possible that it acts as a true vitamin-like entity. The possibility that it acts in the same manner as the known antibiotics is unlikely, since the ration contained aureomycin.

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Reactions of Dextrans with Antiserums of Rabbits Immunized with *S. typhi.** (19132)

JAMES M. NEILL AND IRVING ABRAHAMS.

From the Department of Bacteriology and Immunology, Cornell University Medical College, New York City.

The use of partially hydrolyzed dextrans as plasma volume extenders makes it important to accumulate more complete information on the serological properties of dextrans in general. Previous reports have established that types 2, 20, and 12 pneumococcus antiserums cross react with both the native (unhydrolyzed)(1-9) and the clinical (partially hydrolyzed) products(10). The purpose of this paper is to extend the information on the cross-reacting capacities of these compounds by presenting data on the reactions obtained with antiserums of rabbits immunized with *S. typhi.* Zozaya(11) in 1932 reported cross reactions between a dextran and antiserums of various bacteria, including *S. typhi.* However, Zozaya's evidence is weakened by the facts that the reactions were obtained only with low dilutions of dextran, that only single samples of antiserum were used to represent the cross-reacting bacterial species, and that no controls were included on the possible dextran-reactivity of pre-immunization samples of serum. The validity of the *S. typhi* cross-reaction is established on a better basis by the data in this paper.

Materials. The samples of dextran were supplied by Dr. Edward J. Hehre. The 2 native dextrans (designated *A* and *B*) were prepared by the methods and from the strains of *Leuconostoc mesenteroides* described by Sugg and Hehre(1). The 2 clinical dextrans (designated *C* and *D*) had been prepared for use as plasma volume extenders by controlled acid treatment of native dextrans, and represent partially hydrolyzed products of lower molecular size than native dextrans; they were from different manufacturers and presumably different strains of leuconostoc bacteria had been used in the production process. All the serums were from rabbits. The pre-immunization samples were taken a few days before the first injection and the immune bleedings 6 days after the last injection of vaccine. The immunization consisted of 7 injections at 1 or 2 day intervals over a period of 10 or 11 days. The vaccines used will be indicated in the presentation of the data in Table I.

Experimental. Precipitation tests were made with the 4 dextrans in a series of dilutions ranging from 1:20,000 to 1:2 million against a series of dilutions of all the serums; the test mixtures (0.2 ml of dextran plus 0.2 ml of serum) were incubated 3 hours at 37°C and then stored in the icebox for 5 days. Complement fixation tests were made only with the clinical dextrans; the test mixtures comprised 0.2 ml each of dextran, serum, complement (2 units), and sensitized sheep r.b.c.; 2 hours at 37°C was allowed for the fixation and 1 hour at 37°C for the hemolysis test.

Results. As shown in Table I, the antiserums from all the rabbits immunized with

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* This investigation was supported by a research grant from the National Heart Institute, the National Institutes of Health, Public Health Service.

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TABLE I. Precipitation Reactions of *S. typhi* Antiserums in Tests Against Dextran Solutions.

Immunizing material	Rabbit No.	Highest dilution* of serum that gave precipitation with a series of dilutions of the dextran sol.											
		Native dextran A			Native dextran B			Clinical dextran C			Clinical dextran D		
		20000	200000	2000000	20000	200000	2000000	20000	200000	2000000	20000	200000	2000000
		Vaccines for human prophylaxis†											
S. typhi, S. paratyphi, A, B	1	40	40	10	40	20	10	10	10	—	U	U	U
	2	5	5	U	5	5	U	5	5	—	0	0	0
S. typhi	3,4	10	10	5	10	10	5	5	5	—	U	U	0
	5-8	5-10	5	U-5	5-10	U-5	U	5	U-5	—	0	0	0
		Experimental vaccine‡											
S. typhi	9,10	40-80	20-40	10	80	20	10	10	5-10	U	U	U	U
	11-14	10-20	10-20	5	10	5	5	5	U-5	0	U	0-U	0-U
		Normal serums											
Pre-immunization	2, 9-11	U	U	U-0	U	U	U-0	0	0	0	0	0	0
	Others	0	0	0	0	0	0	0	0	—	0	0	—

* U, undiluted serum; —, no test made; 0, negative reaction with undiluted and diluted serum.

† Bacterial cells from agar cultures, heated at 53°C for 1 hr; both vaccines contained 1 billion *S. typhi* per 1 ml plus 500 million each of *S. paratyphi A* and *B* in case of the polyvalent vaccine; Rabbit No. 1 received total of 6 ml; No. 2 and 3, 10 ml; No. 4, 16 ml; No. 5-8, 18-23 ml. (These vaccines were made by the Laboratories of the N. Y. City Department of Public Health and were kindly supplied to us by Dr. G. I. Steffen).

‡ Bacterial cells from broth cultures of the same strain (779) as in the human prophylactic vaccines, heated at 63°C 30 min.; 1 ml contained approximately 1 billion *S. typhi* per ml; all rabbits in this series received 16 ml, except No. 13, which received 3.5 ml.

S. typhi gave precipitation with the solutions of native dextrans *A* and *B*. There was considerable variation in potency among the antiserums from the different rabbits, which is to be expected in the case of any cross-reaction. However, all the antiserums gave precipitation in 1:5 dilution, and the most potent ones (No. 1,9,10) gave reactions in 1:20 or higher dilution against the 1:20,000 and 1:200,000 dilutions of dextran and in 1:10 dilution against 1:2,000,000 dextran. The fact that undiluted pre-immunization serums of 4 of the rabbits (No. 2, 9, 10, 11) precipitated the native dextrans illustrates the importance of including controls with normal serums, but does not invalidate the significance of the reactions obtained with the immune serums. That the latter reactions were due to antibodies evoked by immunization was shown by absorption experiments in which the capacity to react with the dextrans was removed by treatment of the antiserum with *S. typhi* bacterial cells.†

Both the clinical dextrans also gave precipitation with the typhoid antiserums.‡ But, as shown in Table I, dextran *C* had a higher degree of reactivity than did *D*. That is, *C* gave precipitation with 1:5 dilutions of all the antiserums, whereas *D* reacted only with the more potent antiserums and with them only when the serums were tested undiluted.

Additional evidence of difference between the 2 products was obtained from complement fixation experiments, in which 1:5 to 1:160 dilutions of all the serums were tested against the dilutions of the dextrans given in

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† Although the immunological validity of the cross-reaction seems definitely established by the data, one can not entirely exclude the possibility that the reactions may be due to some accompanying substance rather than to dextran itself. This possibility is being investigated in studies we are making on the serological properties of non-dextran components of leuconostoc bacteria.

‡ The reactions of the clinical dextrans with the antityphoid serums differed from the reactions of the same products with type 2 antipneumococcus serums (Hehre and Sugg)(10) in that the antigen prozone which was prominent with the pneumococcus antiserum was not evident in the case of the antityphoid serums.

TABLE II. Number of Antiserums from 14 Typhoid-Immunized Rabbits* that gave Complement Fixation in Tests with 2 Clinical Dextrans.

		Dilutions of serum					
Dilutions of		5	10	20	40	80	160
Dextran C	20000	14	14	13	6	1	0
	200000	14	14	11	6	4	1
Dextran D	†	0	0	0	0	0	0

* The pre-immunization serums from all the rabbits gave negative reactions with both *C* and *D*.

† Dextran D was tested in 5 dilutions ranging in ten-fold increments from 1:2000 to 1:20000000.

Table II. It is apparent from the data that all of the 14 antiserums gave fixation with dextran *C* but not with dextran *D*; moreover, it should be noted that 6 of the antiserums gave positive reactions with both 1:20,000 and 1:200,000 dilutions of *C* throughout the serum dilution range of 1:5 to 1:40. The results with these 6 more potent antiserums are especially convincing because the difference between the 2 products is manifested over a broad zone of serum and antigen dilution. The question of whether the difference in capacity to react with antityphoid serums was due to a difference in molecular size or to differences in the strains of leuconostoc bacteria used to produce the 2 dextrans was not determined.

However, it was of particular interest to test samples which had shown differences in tendency to give untoward reactions in studies made upon presumably similar groups of people. Five samples which met those requirements were kindly supplied to us by Col. Hinton J. Baker. Samples No. 1 to 4 (from the manufacturer of *C*) had given fairly frequent untoward reactions; Sample No. 5 (from the manufacturer of *D*) had given no untoward reactions up to the time the samples were sent to us.[§] The 5 samples were tested for fixation against our 6 most potent *S. typhi* antiserums, employing the same serum and dextran dilutions as used in the experiment reported in Table II. Samples No 1 to 4 proved essentially the same as *C* in capacity to fix complement, whereas Sample No. 5, was like *D* in giving entirely negative reactions over the entire range of serum and antigen dilutions tested. Thus, in the case of these particular samples, there was some correlation between the tendency to give untoward reactions and the capacity to fix complement with *S. typhi* antiserums.

Other Salmonella. Antiserums produced with *S. oranienburg* and with *S. typhi* strain 42-A-58 (often called Panama No. 58 and commonly used in making human prophylactic vaccines) were also studied. Their reactions with the dextrans were comparable to those reported in Tables I and II for the antiserums of *S. typhi* strain 779, with the exception that the *S. oranienburg* antiserums did not show as sharp a distinction between the 2 clinical dextrans. A number of other Salmonella antiserums,[‖] produced at the Lederle Laboratories for diagnostic use, were tested for capacity to precipitate the native dextrans. Positive reactions were obtained with antiserums produced by immunization with the following species or varieties: *S. paratyphi A var. durazzo*, *S. oranienburg*, *S. berta*, *S. cholerae suis*, and a combined vaccine of *S. thompson* and *S. newport*. The dextran-reactivity was weaker than that of the antiserums in Table I, and in the majority of instances precipitation occurred only with undiluted serum. However, the results were sufficient to indicate that the capacity to evoke dextran-reactive antibodies is possessed by many other *Salmonella* as well as by *S. typhi* and *S. oranienburg*.

Comment. That dextrans[†] produced by *Leuconostoc mesenteroides* react with antibodies evoked by *S. typhi* is of some academic interest simply because of the phylogenetic distance between the 2 species of microorganisms. However, the reactions with the clinical dextrans, which had been prepared for intravenous administration, may be of practical importance. It is noteworthy that, among 5 samples which had been used in studies on humans, the four which had given frequent untoward reactions showed a much greater complement-fixing capacity with S. *typhi* antiserums than did the fifth sample which had given no untoward reactions. This apparent correlation was of course not evidence that the difference in the tendencies to give untoward reactions was due to the difference in the capacities of the samples to react with S. *typhi* antibodies. The data are insufficient to permit definite conclusions. Nevertheless, until evidence to the contrary is obtained, the capacity of clinical dextrans to react with S. *typhi* antiserums is one of the factors to take into account in the use of those products. Theoretically, the importance of this particular factor would vary in different geographical and occupational groups, depending upon the frequency of prophylactic typhoid vaccination and upon the incidence of typhoid infection. With some people, the capacity of dextrans (or of accompanying substances) to cross-react with other antibacterial antibodies might be more important.

[§] These samples (different lot numbers than *C* and *D*) had been used in the studies carried out at the Brooke Army Hospital at Fort Sam Houston, reported by Col. Baker and Col. Pulaski at a conference held by the National Research Council in Washington Jan. 30, 1951. We wish to express our appreciation to Col. Baker for sending the samples and for permission to designate them in this paper as ones that had been used in studies by their group.

[‖] The serums were obtained through the courtesy of Dr. H. D. Piersma and Mr. W. S. Hammond.

Summary. Solutions of dextran, both in the unhydrolyzed or native form and in the partially hydrolyzed or clinical form, gave precipitation reactions with antiserums of rabbits immunized with S. *typhi,* including vaccines prepared for human typhoid prophylaxis. Reactions also occurred with antiserums of some other *Salmonella,* particularly S. *oranienburg*. Among 5 samples of clinical dextran that had previously been used in studies on humans, 4 that had given fairly frequent untoward reactions had greater capacities to fix complement with S. *typhi* antiserums than did the one that had given no untoward reactions.

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Proliferation and Vascularization of Adult Human Epithelium in Subcutaneous Tissues of X-Irradiated Heterologous Hosts.* (19133)

Helene Wallace Toolan. (Introduced by C. P. Rhoads.)
From Sloan-Kettering Institute for Cancer Research, New York City.

Recently a preliminary report was made on the growth and transplantation of human neoplasms in the subcutaneous tissues of x-irradiated rats and mice(1). The present paper describes the proliferation ad vascularization of normal adult human epithelium in similarly treated rats and hamsters. The findings are of interest in that successful transfer of tissues between heterologous species has been considered as possible only when embryonic or malignant tissues are used(2).

Material and methods. Young female albino rats 30-50 days of age (Carworth Farms) were used as hosts in all 6 experiments and 50-70 g female hamsters (*Mesocricetus aurats*) were employed in 3. These animals were of "weanling" age since it had been learned that human tumors could be supported successfully in such x-irradiated hosts for approximately 15 days, in contrast to the 8-10 days reported for older animals (1). Half of the experimental animals were x-irradiated, both the rats and the hamsters receiving a total of 2 doses of 150 r each on alternate days from two 180 kv tubes (one above, one below the animal) with the last irradiation treatment usually on the day prior to implantation. The other half of the ex-

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* We wish to acknowledge support of this study by funds from the National Cancer Institute, Public Health Service, Institution of Research Grants, American Cancer Society and the Damon Runyon Memorial Fund for Cancer Research.

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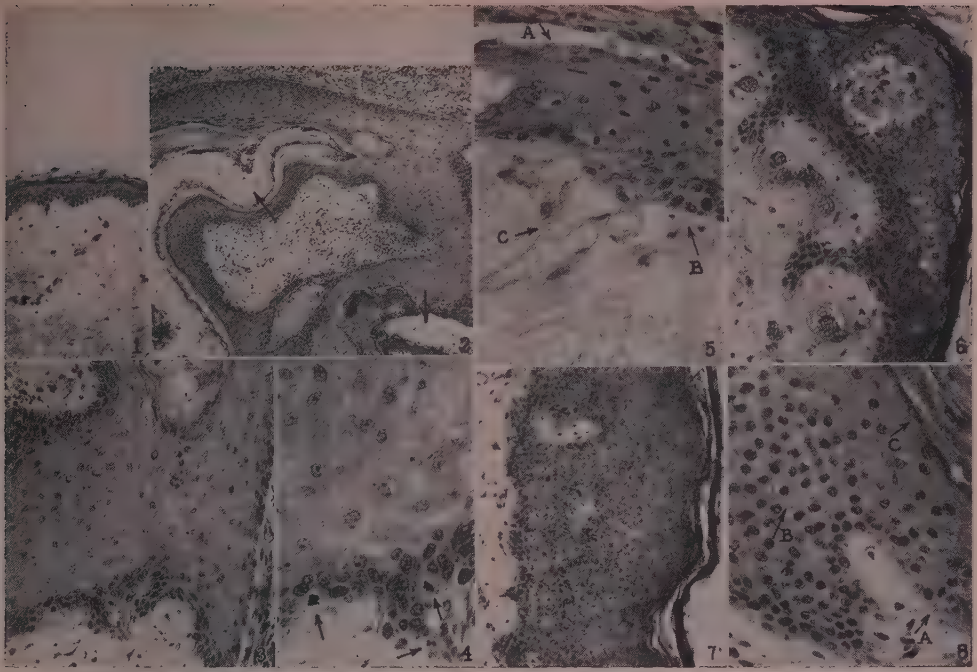

FIG. 1. Original human epithelium, used for one experiment, from chest wall of 63-year-old female. × 80.

FIG. 2. Material from Fig. 1 after 10 days in the subcutaneous tissues of an x-irradiated hamster (arrows point to cysts formed by the epithelium). × 43.

FIG. 3. Magnification of Fig. 2. × 100.

FIG. 4. Detail showing mitotic activity in basal layer (arrows). × 200.

FIG. 5. Four day section of human breast epithelium in x-irradiated rat. Note death of original prickle cells (arrow A). A few basal cells (arrow B) are still alive and associated with a new capillary (arrow C). × 200.

FIG. 6. Six day section of human breast epithelium in an x-irradiated rat. New capillaries are abundant. The basal layer is prominent and in good condition. Sloughing of the original prickle cell layer is still occurring. × 100.

FIG. 7. 10 day section of human breast epithelium (same experiment as Fig. 5 and 6) in x-irradiated rat. Note great increase in thickness of epithelium. × 80.

FIG. 8. 12 day section of human epithelium from chest wall in an x-irradiated rat. The various cell layers are well defined: the basal layer with a mitotic figure (arrow A); the layer of prickle cells with prominent intracellular bridges (arrow B); and the *stratum granulosum* bordering a cyst formed by the epithelium (arrow C). × 200.

perimental animals were normal untreated controls. Human epithelium was obtained in 5 of the 6 experiments from freshly removed surgical material, 2 from the chest wall and 3 from the breast. The sixth specimen was abdominal skin from an autopsy performed 2 hours after the patient's death. Donors were adult females varying from 43 to 72 years of age. Five of these had a breast or rectal adenocarcinoma at a site other than the source of epithelium; a sixth had a benign breast lesion. As soon as the skin was obtained, it was stretched and as much underlying dermis as possible removed by gentle scraping. The epidermis, handled with aseptic technic, was then cut into strips and placed in a petri dish containing sterile Ringer's solution (buffered to a pH of approximately 7.3 with 3 mg of glucose and 300 units of penicillin added per cc) where it was minced with scalpels until the suspension would pass through a No. 18 needle. 0.5 cc of such a concentrated suspension was injected subcutaneously into each flank of the experimental animals. The hamsters received implantations in their cheek pouches(3) as well. In each experiment no less than 18 rats and 12 hamsters (if used) were implanted, so that one normal and 2 irradiated rats and 1 normal and 1 irradiated hamster could be killed at approximately 3-4, 7, 10, 14, 18 and 25 days after the implantation for histological material. There was some variation in the different experiments as to days of killing.

Findings. Though the results of the implantations were most uniform and successful in rats, the overall microscopic findings were the same regardless of the species of experimental animal used. In the case of the hamsters, there was no difference in any one animal, between material implanted in the subcutaneous tissues of the flank and that inserted in the cheek pouch. A general description of the histological sections will therefore be given.

Three days after the minced epithelium had been implanted it was found surviving in comparable condition in both normal and x-irradiated animals. The epithelial fragments with their small amount of attached dermis lay unvascularized and unincorporated in the host tissues. They were often seen forming small cysts—apparently at this time by a process of stretching of the existing cells rather than by mitotic activity since few cells were dividing. The original prickle cells usually were sloughed off or pyknotic and were rarely in good condition. The basal cells, however, appeared to be in fair to excellent shape with an occasional mitotic cell even in the normal un-irradiated animals.

On approximately the fourth day after implantation, host capillaries penetrated the collagen and elastic fibers of the surviving human dermis in the x-irradiated animals (Fig. 5). With one exception (a normal rat killed 4 days after implantation which showed several young host capillaries) the material in the control animals was never vascularized. Though a few epithelial pieces might be found with surviving cells and even with an occasional mitotic figure in the normal hosts as late as the 7th day, by far the majority of the fragments at this time and in these animals were dead. None survived after this period. By contrast, the human epithelium in the x-irradiated hosts was well vascularized (Fig. 6) and actively proliferating on the 6th or 7th day. Mitotic figures were common and appeared to be confined to the basal cells which is of interest in view of Thuringer's(4) opinion that in normal human epidermis the lower one third of the prickle cell layer contains more mitotic figures than the basal layer. By the 10th to the 14th day the fragments of human epithelium had considerably increased their original diameter (Fig. 1), often as much as tenfold (Fig. 2-4, 7 and 8). It should be noted that here and there among the large cysts "nests" of epithelium were often seen, made up of small groups of cells which had become separated during the implantation.

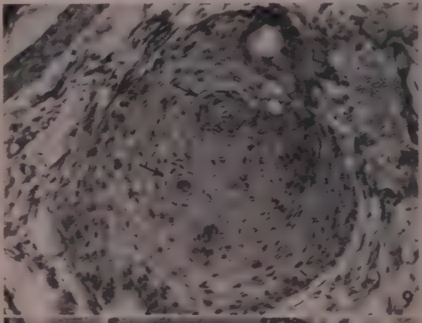

FIG. 9. Typical small "nest" of human epithelial cells in an x-irradiated rat 8 days after implantation. Attempts to form cysts by such a small group of cells produces the "perls" seen here (arrows). × 100.

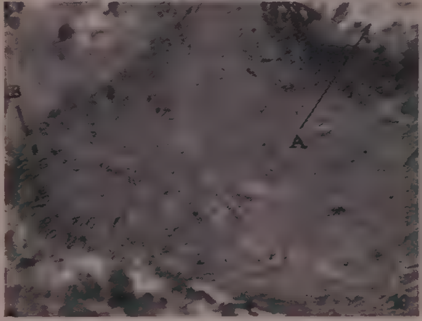

FIG. 10. Human epithelium originally from the chest wall (Fig. 1) after 3 generations (31 days) in x-irradiated rats. Cysts are still being formed (arrow "A"). Arrow "B" points to a mitotic figure in basal cell layer. × 300.

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These cells, in attempting to form the usual cysts, often produced what appeared to be typical "perls" instead (Fig. 9).

At approximately 14-16 days, the implanted tissues in the irradiated animals apparently died quite suddenly. In all the 18 and 25 day sections the human epithelium was dead. The cell nuclei were entirely pyknotic, the basal layer separate and shrunken away from the underlying and remaining dermis, and the whole invaded by host cells, particularly polymorphonuclear leukocytes. Lymphocytes, though present, were not conspicuous. Many of the "new" capillaries contained an amorphous basophilic material, not identified. Considerable pigment was found among the basal cells of these late sections. (A small amount had been seen in the 12 and 14 day tissues).

Transplantation. In 4 experiments the material found at the implantation sites in x-irradiated rats killed 10 days after injection, was pooled, minced and re-implanted in other x-irradiated rats. Viable epithelial tissue was found in this second group of irradiated animals 11 days after the implantation in three of the 4 transfer attempts. A second transfer was made of the 3 surviving tissues to a 3rd group of irradiated rats. Two of the three transfers had vascularized epithelial cysts with cells in mitosis 10 days later. Fig. 10 shows such cells 31 days after the original material was obtained from a human patient and more than 2 weeks after the implanted material had ceased to survive in the original group of x-irradiated animals. No transplants beyond the third generation were attempted.

Discussion. Recently considerable interest has been aroused in the intraocular heterologous transplantation of human tissue, since Greene and his coworkers(2) have maintained that such transplantation is a diagnostic test for malignancy. It is the premise of these workers that only embryonic tissues and tumors which have attained a high degree of malignancy can be grown with success heterologously. In view of this opinion, it is noteworthy that the experiments herein described, show that normal human epithelium as well as tumors can become vascularized and can increase as much as tenfold in the subcutaneous tissues of x-irradiated heterologous hosts, and that, by transfer into new groups of these animals, both the normal and malignant human tissues can be maintained in "healthy" and proliferating condition.

The success or failure of the human tissue to grow does not appear to depend upon its autonomy but rather to be a function of the heterologous host resistance after alteration by previous x-irradiation. It should be noted that other normal or benign human tissues, such as colloid goiter, can be grown and transferred, in flourishing condition, in the subcutaneous tissues of x-irradiated heterologous hosts as will be reported in a later paper.

Summary. In 6 experiments, minced fragments of normal adult human epithelium implanted subcutaneously in previously x-irradiated heterologous hosts (rats and hamsters) became vascularized, proliferated and increased for approximately 15 days. After this period they died suddenly. In un-irradiated control animals the epithelial material survived in poor condition for 3-7 days but did not become vascularized. When transferred every 10 days to new groups of x-irradiated animals, human epithelium remained viable and in active mitosis for the duration of the experiment (3 generations or 31 days).

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Blood Volume of Swine.[*] (19134)

Sam L. Hansard, H. E. Sauberlich,[†] and C. L. Comar.

From the University of Tennessee-Atomic Energy Commission Agricultural Research Program, Oak Ridge, Tenn.

Blood volume values for the various species of animals have been reported and summarized by numerous investigators(1,2). So far as is known, however, no such values for swine have appeared in the literature. This paper, therefore, presents blood volumes for healthy growing swine of various body weights as determined by the P^{32} labeled red blood cell method.

Methods. Many procedures for the determination of plasma volume, red blood cell volume and total blood volume have been reported and compared(3-6). The method used in this study was a modification of that discussed in considerable detail by Reeve and Veall(6). The procedures for handling the animals and performing the injections have been previously described(7,8). Precautions were taken where necessary to prevent damage to the red blood cells, such as gentle handling and the use of heparinized containers and syringes. From the restrained animal, 5 to 10 ml of blood was taken, heparinized and placed in a 50 ml Erlenmeyer flask. Fifty to 100 microcuries of P^{32} in less than one ml of neutralized isotonic saline was added and the blood incubated at 37.5°C for 2 to 4 hours. The blood was centrifuged and the red blood cells washed 4 or 5 times with ice-cold physiological saline which removed all but insignificant amounts of the radioactive plasma. The labeled cells were then reconstituted to the original volume of blood with saline, and, depending upon the size of the animal, a measured volume of from 2 to 5 ml was quantitatively injected intravenously. Venous samples were drawn at 15 minutes after injection. A one ml aliquot of the cell suspension injected was diluted to 250 ml for the standard, and the whole blood sample was usually diluted fivefold for the measurements. The radioactivity was measured with a Geiger counter arranged for external solution counting(9). Cell pack volumes were determined by centrifugation in the usual manner and were corrected for 5% trapped plasma. Since the labeled red cells were usually injected into the animal within 15 minutes of the preparation of the final suspension, no correction was made for free P^{32} in the saline. Blood volumes are expressed as milliliters of blood per 100 g body weight.

In vitro uptake and in vivo loss of P^{32} from porcine red blood cells: It is helpful to be

TABLE I. Uptake of P^{32} by Porcine Red Blood Cells *In Vitro.*

Time, hr	% uptake
.5	10
1	15
1.5	18
2	20
3	22
4	23
4.5	24

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[*] Published with the approval of the Director of the Tenn. Agric. Exp. Station. Radioactive materials were obtained from the Oak Ridge National Laboratory on allocation from the U. S. Atomic Energy Commission.

[†] Oak Ridge Institute of Nuclear Studies Fellow on leave from Alabama Polytechnic Institute.

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TABLE II. Blood Volume of Swine.

Pig No. and sex	Body wt, lb	Blood vol value*	Cell pack vol
1 ♂	10	7.9	34
2 ♂	11	7	40.2
3 ♀	8	7.3	34
Avg	10	7.4	35
4 ♂	15	6.6	40.2
5 ♂	21	6.6	40.9
6 ♀	20	6.7	42.3
7 ♀	22	6.9	40.6
Avg	20	6.7	41
25 ♀	74	6	31.8
26 ♀	82	5.6	37.8
26 ♀	72	5.9	38.8
27 ♀	78	6.4	37.9
Avg	76	6	36
31 ♂	94	6.6	41.9
28 ♂	97	6.5	43.1
28 ♂	97	6.8	39
30 ♂	102	6.1	30.3
Avg	98	6.5	39.1
31 ♂	134	6.5	37.9
29 ♂	118	5.3	38.2
3 ♂	113	5.9	39.3
Avg	122	5.9	36.8
29 ♂	182	4.6	40
49 ♂	164	6.1	39.3
0 ♀	200	4.9	39.3
Avg	182	5.2	39.5
37 ♂	370	4.4	36.9
45 ♀	333	4.3	38.6
45 ♀	328	4.6	39.7
0 ♀	344	5.1	38.5
Avg	344	4.6	38.4

* ml of blood per 100 g body wt.

able to approximate the percentage of added P^{32} that will enter the cells under the conditions of the experiment. Table I shows the percentage of P^{32} found in the cells after varying periods of incubation of the normal whole blood with P^{32}. The percent uptake leveled off at about 20% at 2 hours. Experiments have indicated that in the final suspension the loss of P^{32} from the cells into the saline was of the order of 5% during the first hour. After injection of the labeled red cells into the animal it was found that the loss of P^{32} during the first hour was approximately 10 to 12%. The loss during the 15 minutes after injection was not found to be significant. These findings indicate the suitability of the method for blood volume estimation in swine.

Results and discussion. Twenty-five determinations were made with 16 Hampshire swine as indicated in Table II: the animals of each group were litter mates. The cell pack volumes have been listed to permit estimation of the plasma and red cell volume. An average blood volume of 7.4 ml/100 g body weight was obtained with the 10 lb pigs, with the values decreasing more or less regularly to an average of 4.6 for the 344 lb animals. Consideration of the data indicated that despite the variation between individuals there was a real decrease of the blood volume value with increasing body size; furthermore, the effect was more pronounced with the lowest weight groups.

Dukes(1) has reported the blood volume value to be independent of body weight in dogs weighing between 7 and 24 lb and in goats between 16 and 67 lb. However, it was also noted by Dukes that blood values in lambs did decrease with age between birth and 190 days with the greatest decrease occurring in the first 30 to 60 days.

In working with swine perhaps the greatest uncertainty is in obtaining a quantitative injection. In this study there were injection losses in four animals, as evidenced by observations during the procedure, which always resulted in blood volume values 50 to 100% higher than expected on the basis of other determinations. These animals were not included in the data presented.

Summary. The P^{32} labeled red blood cell method has proven satisfactory for the determination of blood volumes in swine. Values obtained ranged in regular fashion from 7.4 to 4.6 ml/100 g body weight for 10 to 344 lb animals, respectively.

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A New Method for Studying Mechanics of Breathing Using Cathode Ray Oscillograph.* (19135)

A. B. DuBois† and B. B. Ross. (Introduced by W. O. Fenn.)

From the Department of Physiology and Vital Economics, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

It is the purpose of this paper to describe the use of the cathode ray oscillograph for the direct recording of pressure-volume or pressure-flow relationships. In addition, the use of a catheter for measuring alveolar pressure and the use of the oscilloscope for studying lag and phase shifts due to the inertia of the chest are described.

Apparatus. The equipment used is as follows: Two differential pressure gauges (Statham) with control unit (Hathaway); a cathode ray oscilloscope (Dumont 304-H); a recording spirometer equipped with a potentiometer geared to the pulley to act as a volume transducer; an Electrodyne Stimulator used as a time marker which interrupted the cathode ray beam at 0.1 sec. intervals; a pneumotachometer constructed of a porous bronze disc; a Drinker respirator for generation of cyclical pressures used in some experiments; and a 35 mm camera with bulb exposure and light shield for photographing the face of the oscilloscope. The arrangement of the apparatus was varied depending on the particular relationship studied, and will be described with each type of experiment.

Pressure-volume curve of the chest. The subject is connected by a tight-fitting mask to a rebreathing circuit including the recording spirometer. The subject may be inside the respirator with his head and the spirometer outside or, more conveniently, the spirometer may be inside communicating through tubes to the subject outside. In either case the subject endeavors to relax completely and permits the respirator to supply all the pressure required for the expansion and collapse of the chest. Alternating positive and negative pressures of varying magnitude and frequency are produced in the respirator. Pressure is recorded at the mouth by a strain gauge which produces a potential causing the beam of a cathode ray oscilloscope to move horizontally. Volume is recorded from the spirometer which produces a similar potential causing the spot to move vertically. With this arrangement a single respiratory cycle is represented by a loop on the oscilloscope. The record is interrupted at 0.1 sec. intervals by rhythmical potentials from the Electrodyne Stimulator applied to the Z axis of the oscilloscope. In Fig. 1A, 3 such loops at different pressure ranges were recorded on the same film by opening the camera shutter during 3 separate cycles. Inspiration is recorded upwards and positive mouth pressure, cm H_2O, to the left. A straight line, EL, drawn through the points of maximum and minimum volume on these loops represents the elastic component of the total pressure. Additional force, represented by a horizontal line from the elastic component line, EL, to the curve, is required to overcome resistance to the flow of gases through the tracheo-bronchial tree and tissue "viscance." This additional force is the *dynamic component* of the work of breathing and is a function of the rate of change of volume. In Fig. 1A the rate of change in volume is indicated by the vertical increment between the 0.1 second interval marks. The total dynamic work of breathing is represented by the area of the loop.

Resistance to flow is the ratio of the dynamic pressure (horizontal distance from EL to the curve) to the rate of change in volume, (vertical distance between successive spots). Tissue viscance is also included in this measurement. A rapid estimate of "conductance" (the reciprocal of resistance) during any time interval may be made by drawing a horizontal line from one point on the loop to the line EL

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† Fellow of the Life Insurance Medical Research Fund.

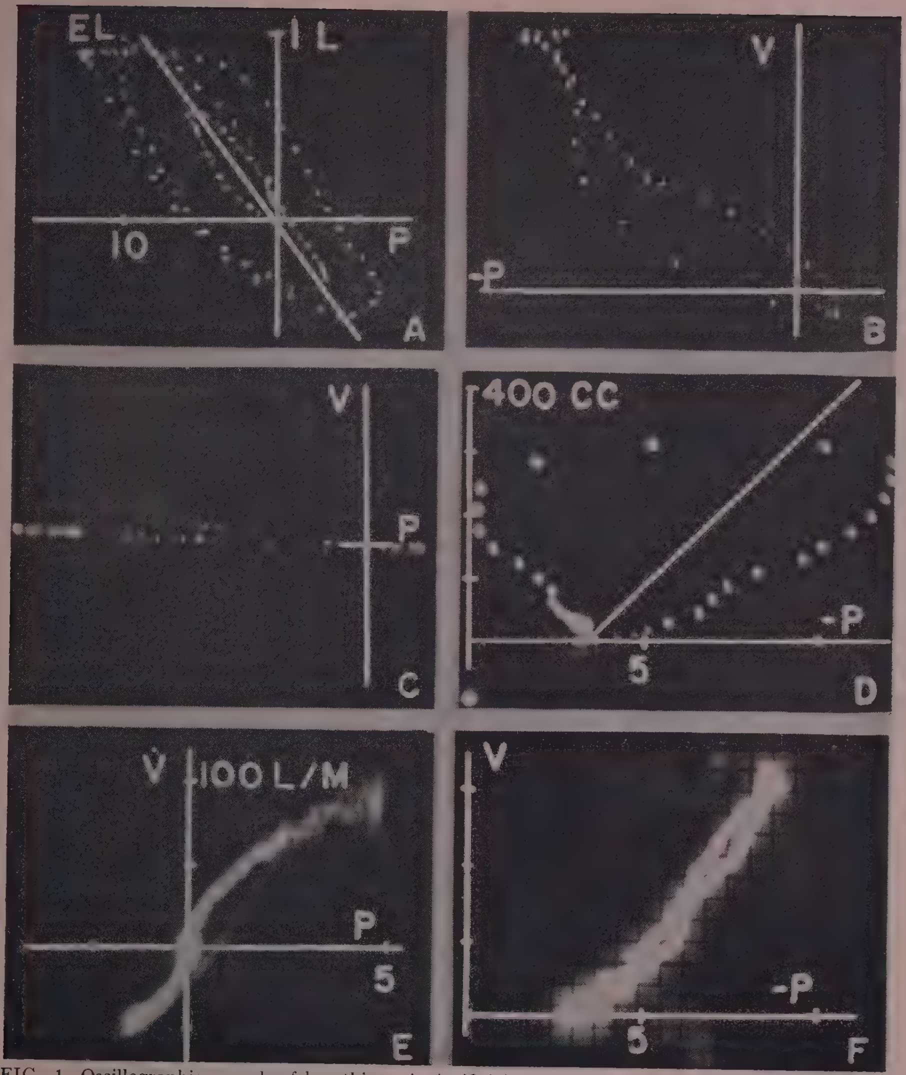

FIG. 1. Oscillographic records of breathing. A. Artificial respiration in the human subject—3 cycles at increasing pressures. Transthoracic pressures vs. volume. B. The same in a human poliomyelitis patient who is actively trying to assist the respirator. C. The same while the patient resists the respirator. D. Intrapleural pressure vs. volume in a dog. E. Alveolar pressure (P) vs. gas flow (liters per minute) in a dog. F. Lung tension (alveolar minus intra-pleural pressure) in cm H_2O vs. volume (V) in a dog. Marks on Y axis represent 100 cc increments.

and connecting the point of intersection to the next point on the loop. The angle thus subtended by two adjacent points is (nearly) proportional to the conductance. This method of analysis is useful in comparing different loops or different parts of the same loop.

Deviations from the normal pressure-volume curve occur when there is a change in tone of the chest muscles, or a change in resistance to gas flow or tissue movement. With respect to these possible deviations there is a double advantage in using the cathode ray oscilloscope. One may ascertain immediately whether the subject is relaxed and is following the respirator merely by observing the curve as it is generated. On the other hand, chest function may be tested by asking the subject to aid or resist the action of the respirator deliberately. In either case, part of the dynamic work of breathing will not appear because it has been replaced by muscular force which cannot be measured in terms of pressure. This is illustrated in Fig. 1B which was obtained by asking a poliomyelitis patient with an indwelling tracheotomy tube to aid the respirator as much as possible. The patient followed the action of the respirator until a point near maximum inspired volume was reached, whereupon a rapid inspiratory effort was made. The patient then relaxed, again following the respirator during expiration.

Fig. 1C shows the result obtained when the same patient was asked to resist the respirator. Only a small volume change was brought about, indicating that the patient had retained some control of his respiratory muscles.

Intrapleural pressure-volume curve. Transpulmonary pressure (between mouth and pleural space) is measured by a differential strain gauge and transmitted to the X axis of the oscilloscope. Volume is recorded as in the previous method. The 0.1 sec. intervals are brought about as before through the Z axis of the oscilloscope. This approach is illustrated in Fig. 1D, obtained from an anesthetized dog. In this case, the curve is plotted counterclockwise, originating at the abscissa at the point of negative pressure normally existing in the pleural space during the respiratory pause. Pressure is noted in terms of cm H_2O and lung volume in 100 cc units. The line of elastic force (lung tension) is drawn through the points of maximum and minimum volume. This is difficult to do precisely in Fig. 1D because of the rapid change in pressure without apparent change in volume at the height of inspiration. This seems to be a characteristic of the respiratory pattern of the dog. In this case the selection of the proper point was facilitated by simultaneous time records of changes in pressure and volume taken for another purpose. This permitted the selection of the pressure simultaneous with the point of maximum volume.

Alveolar pressure and gas flow. A Cournand cardiac catheter connected to a strain gauge is introduced into the bronchial tree of an anesthetized dog and advanced until it becomes occluded in a small bronchus.[1] The small segment of lung thus occluded presumably derives its pressures from the surrounding lung, yielding a satisfactory approximation of alveolar pressure. This pressure is transmitted to the X axis of the oscilloscope and gas flow is applied to the Y axis by means of a pneumotachometer (Fig. 1E). During inspiration the curve starts at the origin, moves down and to the left and then returns to the origin along the same curve. During expiration it moves up and to the right in the opposite quadrant, again returning to the origin. These two curves are represented by the equation $P = K\dot{V} + K'\dot{V}^2$(1). The constants K and K′ are easily derived by plotting points obtained from the curves according to the equation $P/\dot{V} = K + K'\dot{V}$. In the figure shown, these constants are $K = 0.3$ and $K' = 0.5$ (when $P =$ cm H_2O and $\dot{V} =$ l/min.). The inspiratory and expiratory curve are identical when superimposed and indicate that in this animal there is no appreciable change in the constants K and K′ with respect to the phase of the respiratory cycle. Loops are sometimes observed which are due to mechanical factors such as intermittent occlusion of the catheter or leakage around the tip of the catheter when the bronchi are fully dilated by inspiration. These are easily eliminated by repositioning the catheter.

Lung tension curve. A differential strain gauge is connected to an intrapleural needle and an alveolar catheter in a dog and led to the X axis of the oscilloscope. This arrangement effectively subtracts the air flow component of intrapleural tension from the total, leaving only the elastic component of lung tension. Volume at the mouth is recorded on the Y axis by means of a spirometer. The resulting curve is shown in Fig. 1F. During inspiration the curve moves up and to the right. During expiration it returns toward the starting point. The slope of this line represents the extensibility of the lung, or change in volume per unit pressure. This is the inverse of the elastic constant (more strictly, the volume modulus). Deviations from this line occur when there is partial obstruction of the needle or catheter. These are cleared with an air syringe. Theoretically, there should be a small loop representing work in overcoming tissue viscance. The figure presented shows a very slight deviation but this is obscured by other irregularities which are presumably due to heart action. The slope of this curve is nearly the same as the one drawn in Fig. 1D.

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[1] Otis, A. B., Fenn, W. O., and Rahn, H., *J. App. Physiol.*, 1950, v2, 592.

Lag between pressure around chest and at mouth. For the purposes of ordinary testing of pressure-volume and pressure-flow relationships, it is convenient to assume that the inertia of the chest and gases is negligible. To test this assumption a subject was placed in the respirator, and a sudden pressure was released into it from a 3-liter can containing air compressed to 7 lb per square inch above atmospheric pressure. This triggered a single sweep on the oscilloscope, and pressure at the occluded mouth of the subject was recorded on the Y axis. The pressure in the respirator reached maximum in about a sixtieth of a second, and the pressure at the mouth of the subject reached maximum within two-sixtieths of a second. This closed chest-mouth system thus showed a small but finite lag due presumably in large part to inertia of the chest. A small transmission time from alveoli to mouth is included.

Natural frequency of the chest. The lag due to inertia of the chest could be expected to cause a shift in phase between a rapidly oscillating transthoracic pressure and the resultant flow at the mouth. A cylinder and piston whose shaft was eccentrically placed on a wheel were connected to the respirator. Pressure fluctuations which are sinusoidal in nature were produced up to a frequency of 20 per second. Transthoracic pressure was measured by a strain gauge and transmitted to the X axis, and flow at the mouth by a pneumotachometer which drove the Y axis of the oscilloscope. As the frequency of the piston was increased to 15 cps the resulting curve widened out toward an elliptical form. The presence of inertia of the chest was thus demonstrated. Vibration of the apparatus became excessive at frequencies above 20 cps. The inertia of the chest is of an order of magnitude which makes it unnecessary to consider it in an ordinary analysis of the mechanics of breathing. However, it may be of significance in unusual situations such as explosive decompression. It is suggested that the cathode ray oscilloscope provides a convenient means of visualizing this factor.

Summary. (1) Pressure-volume curves of the chest and lungs may be portrayed directly on the cathode ray oscilloscope to show the elastic and viscous properties described by previous authors(1,2). Alterations of muscle tone of the thorax affect the pressure-volume curve in such a way as to aid or resist artificial respiration and are immediately detectable by this method. (2) Pressure-flow curves may be recorded in a similar manner. This method permits the easy evaluation of the constants involved in the equation $P = K\dot{V} + K'\dot{V}^2$ which are determined by the physical characteristics of the lung tree. (3) Inertia of the thorax causes a small lag between pressure around the chest and flow at the mouth. This time lag is of the order of one-sixtieth of a second.

The authors wish to gratefully acknowledge the advice and help of Dr. W. O. Fenn and Dr. H. Rahn throughout the experiments.

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Recovery of Growth Hormone in Purification of Corticotropin. (19136)

M. S. Raben and V. W. Westermeyer.* (Introduced by E. B. Astwood.)

From the Ziskind Research Laboratories, New England Center Hospital, and the Department of Medicine, Tufts College Medical School, Boston, Mass.

Current procedures for the extraction and purification of pituitary growth hormone have given consideration to the reported ready destructibility of the active principle(1), consistent with estimations of a large molecular size and protein character(2,3). Sensitivity to heat(1,2,4,5) and to acid(2) have been noted and most procedures have been performed in alkaline or near-neutral media (1-3,6,7), and sometimes in the cold(1,3). It is perhaps surprising, therefore, to find that the method recently reported by Astwood, Raben, Payne, and Grady(8) for the purification of the highly acid-resistant corticotropin leaves in the final residual solution an abundance of active growth hormone.

Initial extraction of acetone-dried pig anterior pituitary powder with glacial acetic acid at 70°C was followed by removal of an acetone precipitate containing thyrotropin, gonadotropins, and some luteotropin; crude corticotropin was then obtained by precipitation with ether(9). The crude corticotropin, with a potency of about 2 u/mg, was dissolved with warming in 0.1 N acetic acid and stirred with 8% its weight of powdered oxidized cellulose (10.4% COOH) for 20 hours at room temperature. The oxycellulose removed 2% of the solids of the pituitary extract and 90% of the corticotropic activity. The remaining acetic acid fraction, containing about 12% of the solids of the original acetone-dried pituitary powder and 98% of the solids of the crude corticotropin, was found to be rich in growth hormone. Retreatment of this fraction with a 20% weight of oxidized cellulose removed almost all the residual corticotropin without detectable loss of growth hormone. When tested in hypophysectomized Sprague-Dawley male rats,† weighing 80 to 100 g, by the weight gain produced with daily intraperitoneal injections, this fraction appeared to be roughly 20% as active as the purified growth hormone preparation of Wilhelmi (Table I). Adjustment of the pH of the acetic acid fraction to 5.5 in the presence of 0.3 N potassium acetate caused 78% of the material to precipitate from a 2.5% solution. The precipitate (No. 4, Table I) was about as active as the whole acetic acid fraction. Similarly, precipitation of 70% of this fraction at pH 3.5 did not effect a separation of growth activity from the major portion of the solids (No. 3, Table I). Purification was achieved, however, with a modification of Wilhelmi's procedure(3), by removing the precipitate formed at pH 8.5, and adding 95% ethanol to the supernatant to 47.5 or 60% concentration. The alcohol precipitate containing 10.5 to 13% of the solids was approximately as potent as the purified preparation used for reference (No. 6, Table I).

Growth hormone had a relatively low affinity for oxycellulose and the cellulose appeared to be a poor fractionating agent for growth hormone when employed in the manner described. Comparing the adsorption of

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* Fellow, New England Chapter, Arthritis and Rheumatism Foundation.

† Some of the animals used were hypophysectomized by the Charles River Breeding Laboratory, Boston.

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TABLE I. Growth Effect as Tested by Daily Intraperitoneal Injections in Hypophysectomized Rats.

No.	Substance tested	No. of rats	Test period, days	Daily dose, µg	Avg wt gain per day, g
1	Acetic acid fraction	5	9	100	.9
		3	11	250	1.3
2	a. 16.5% of (1) eluted from oxycellulose	3	11	200	1.6
	b. Unabsorbed portion	5	10	100	1
		5	4	500	2.3
3	pH 3.5 ppt.—70% of (1)	2	6	250	1.5
4	pH 5.5 ppt.—78% of (1)	5	10	250–2d. 100–8d.	1.1
5	pH 8.5 ppt.—57% of (1)	7	13	100	.3
		7	7	250	.4
6	Ethanol ppt. of pH 8.5 soluble—10.5% of (1)	11	10	30	1.1
7	Wilhelmi growth hormone	3	10	25	1.1
		4	10	30	.7

an electrophoretically homogeneous growth hormone preparation(3), kindly provided by A. E. Wilhelmi, with that of purified corticotropin, it was found that the amount adsorbed on 1 g of oxycellulose (10.4% COOH) from a 0.1 N acetic acid solution, and eluted with 0.1 N hydrochloric acid, was approximately 6 mg of the growth hormone, and 340 mg of corticotropin(10). Treatment of the acetic fraction with a 12.5-fold weight of 19% carboxyl oxycellulose yielded only 16.5% of the material in the eluted fraction. The growth activity, furthermore, appeared to be about equally concentrated in the eluted and the unadsorbed fractions (No. 2, Table I).

Studies on the stability of growth hormone have led Li to conclude that growth hormone is more stable in alkali than acid(11). This is in contrast to earlier work which had indicated to Evans, Meyer, and Simpson that the hormone was more susceptible to alkali(1), and to Collip that growth hormone was most stable at pH 2(4). The preparations described here survived a glacial acetic acid extraction at 70°C and solution in 0.1 N acetic acid for 2 days at room temperature and for 5 to 6 weeks at 10°C.

Kamm and coworkers reported that the posterior lobe principles could be extracted with glacial acetic acid at 25°C but that the extraction was facilitated by the presence of 2% water(12). Evans, Meyer, and Simpson found the growth hormone to be extractable at 20°C with 95 to 98% acetic acid, but not with glacial acetic acid(1). It has recently been shown that the presence of as little as 0.5% water improves the efficiency of extraction of corticotropin with acetic acid at room temperature(13). The glacial acetic acid used for the extraction of corticotropin (14,9,8), and as used in the present work, was standard reagent grade acid, stated to contain about 0.5% water.

Summary. Extraction of acetone-dried hog anterior pituitary glands with glacial acetic acid at 70°C and removal of corticotropin with oxycellulose provides a method for the preparation of corticotropin and growth hormone in a single procedure.

We are indebted to Dr. David Klein of the Wilson Laboratories for a generous supply of pituitary powders.

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Effects of Chorionic Gonadotropin on Sex Organs of Male Rats Deficient in Essential Fatty Acids.* (19137)

S. M. Greenberg and B. H. Ershoff.

From the Department of Biochemistry and Nutrition, University of Southern California, and the Emory W. Thurston Laboratories, Los Angeles.

Considerable data are available indicating that testicular function is impaired in rats deficient in the essential fatty acids. Burr and Burr(1) observed that male rats on fat-free diets were unable to sire normal young, that they did not usually copulate and that the occasional copulation that did take place was infertile. Similar findings have been reported by Evans *et al.*(2), Quackenbush *et al.*(3), and others. Histologically the testes of rats fed a fat-free diet for approximately 5 months revealed complete absence of sperm and a marked degeneration of the seminiferous epithelium(2). Testicular dysfunction as judged by failure of sperm formation or atrophy of the secondary sex glands has also been observed in rats deprived of other nutrients including thiamine, riboflavin, pyridoxine, pantothenic acid, biotin, protein and others, as well as the fat-soluble vit. A and E. It would appear that in the case of at least some of these nutrients testicular dysfunction was due to an inadequate secretion of pituitary gonadotropin (resulting either from the nutritional deficiency *per se* or the attendant reduction in caloric intake). This is indicated by the fact that injections of chorionic gonadotropin restored normal structure and function in the testes of rats deprived of thiamine (4,5), pyridoxine(5), pantothenic acid(5), or protein(5). Gonadotropin stimulation, however, failed to promote spermatogenesis in rats depleted of vit. A(6) or vit. E(7-9), although it did correct the atrophic changes in the prostate and seminal vesicles of A-depleted rats(6,10,11). In the present communication data are presented on the effects of chorionic gonadotropin on the testes and secondary sex glands of male rats maintained for a prolonged period of time on a diet deficient in essential fatty acids.

Procedure and results. The basal ration employed in the present experiment consisted of sucrose, 72%; casein,† 20%; salt mixture,‡ 4%; cellulose,§ 4%; and the following synthetic vitamins per kg of diet: Thiamine hydrochloride, 72 mg; riboflavin, 72 mg; pyridoxine hydrochloride, 27 mg; calcium pantothenate, 67 mg; nicotinic acid, 10 mg; i-inositol, 500 mg; para-aminobenzoic acid, 200 mg; folic acid, 10 mg; 2-methyl-naphthoquinone, 5 mg; d-biotin, 1 mg; and choline chloride, 1.2 g. Each rat also received twice weekly oral supplements of the following fat soluble vitamins dissolved in propylene glycol (0.1 ml): 158 U.S.P. units of vitamin A,‖ 15.8 U.S.P. units of vitamin D,‖ and 2.8 mg of alpha-tocopherol. Twenty-four male rats of the University of Southern California strain were selected at 21 to 23 days of age for the present experiment. Animals were kept in metal cages with raised screen bottoms to prevent access to feces and were fed the above diet *ad lib.* for a period of 12 weeks. At this time evidence of essential fatty acid deficiency was observed in all rats as judged by failure to gain weight for a period of at least 3 weeks and dermatitis of the paws and tail. Rats were then divided into 3 groups, all of which were continued on the basal ration, but which received, in addition, the following supplements administered orally by graduated syringe 4 times weekly: Group I, 175 mg methyl linoleate¶ in 0.3 cc ethyl laurate (12 rats); group II, 8.75 mg methyl linoleate¶ in 0.1 cc ethyl laurate (6 rats); and group III, 0.1 cc ethyl laurate alone (6 rats). After 8 weeks of supplementation the body weight of rats in group I averaged 260.3 g (range 193 to 339 g) and all manifestations of essential fatty acid deficiency had been ameliorated. Groups II and III average 182.7 and 176.0 g respectively (range 111 to 225 g) and dermatological symptoms characteristic of essential fatty acid deficiency were marked in all rats. The level of essential fatty acids fed to group II was extremely low. Previous investigations from this laboratory(12,13) indicate that the optimum dosage of methyl linoleate for growth under conditions similar to those employed in the present experiment is from 20 to 40 times greater than the level fed to group II. Since no significant difference was observed either in the weight or gross appearance of rats in groups II and III, and since no difference was observed in the response of these groups in the present experiment, these groups will henceforth be reported and discussed as a single (negative) group in the present report. After 8 weeks of supplementation each group was divided into 2 parts. Half the animals in each group received 7 daily subcutaneous injections of 20 International Units of chorionic gonadotropin.** The other half were similarly treated except that saline solution was employed. The supplements fed for the preceding 8 weeks were continued for the duration of the experiment. All rats were autopsied 24 hours after the last injection. Testes, prostate and seminal vesicle weights were determined; testes were fixed in Bouin's solution, and sections were prepared and stained with hematoxylin and eosin.

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‡ Osborne-Mendel Salt Mixture.

§ Celluflour, Chicago Dietetic Supply House, Chicago, Ill.

‖ Nopco Fish Oil Concentrate, assaying 800000 U.S.P. units of vit. A and 80000 U.S.P. units of vit. D per g.

Findings are summarized in Table I. No significant difference in testes weight was observed in the various groups regardless of whether chorionic gonadotropin or saline solution was administered. Gonadal weight was somewhat larger for rats in group I but when expressed as per cent of total body weight comparable values were obtained for all groups. Furthermore, no significant difference was observed histologically between the testes of rats fed a diet deficient in essential fatty acids and that of animals in group I or of rats of similar weight which had been fed a natural food ration. Mature sperm were present in the tubules of all rats and no evidence of seminal epithelial degeneration was observed in any of the rats fed a diet deficient in essential fatty acids. These findings are in contrast to those of Evans *et al.*(2), who reported complete absence of sperm in the testes of rats deficient in essential fatty acids but are consistent with the observation of Mackenzie *et al.*(14) that motile sperm were present in the epididymides of rats after 44 weeks on a low fat dietary regime. Both the prostate and seminal vesicle weights of deficient rats injected with saline were significantly less than that of comparable animals in group I. Administration of chorionic gonadotropin restored the weight of these secondary sex glands to normal values. Chorionic gonadotropin also increased the prostate and seminal vesicle weights of rats in group I, but to a smaller degree than in the deficient rats.

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TABLE I. Effects of Chorionic Gonadotropin on Testes and Secondary Sex Glands of Male Rats Deficient in Essential Fatty Acids. Six animals per group.

Dietary group	Final body wt, g	Testes wt, g	Prostate wt, mg avg	range	Seminal vesicle wt, mg avg	range
		Saline series				
Deficient rats	198.8	2.222	165	(122-215)	510	(256-873)
Linoleic supplemented controls	268.5	2.679	249	(208-294)	1154	(979-1419)
		Chorionic series				
Deficient rats	182.8	1.843	353	(243-466)	959	(452-1903)
Linoleic supplemented controls	273.2	2.573	378	(315-428)	1390	(1090-1869)

Results of the present experiment suggest that the reduction in prostate and seminal vesicle weights of rats fed a diet deficient in essential fatty acids was due to insufficiency of pituitary gonadotropin (interstitial cell stimulating hormone) secretion. This is indicated by the fact that gonadotropin stimulation restored the weight of these organs to normal. Available data, however, do not indicate whether this insufficiency was absolute or relative and to what extent, if any, the sensitivity of the interstitial cells of the testes or the target organs of its secretion may have been altered as a consequence of essential fatty acid deficiency. Caloric restriction *per se* does not appear to be the cause of the deficient gonadotropin secretion inasmuch as the food intake of rats fed the deficient diet was not less than that of animals in group I. It is possible, however, that the reduced efficiency of food utilization in the deficient rats resulted in an inanition effect despite the apparent adequacy of calories consumed.

Summary. Rats deficient in essential fatty acids exhibited a marked reduction in prostate and seminal vesicle weight. Administration of either methyl linoleate or chorionic gonadotropin restored these organs to normal weight.

The authors are indebted to Miss Lilla Anisfeld for her technical assistance.

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Aureomycin and Terramycin in Human Saliva.* (19138)

FREDERICK W. KRAUS, DIANA W. CASEY, AND VILMA JOHNSON.
(Introduced by David Rapport.)
From Tufts College Dental School, Boston, Mass.

Many pathogens multiply in the oral cavity before spreading further. Antibiotics present in saliva might conceivably be effective in preventing the spread of microorganisms. However, little is known concerning the mean concentrations of antibiotics excreted in saliva of human subjects, and the changes in levels with time. Abraham *et al.*(1), Struble and Bellows(2), and Cutting *et al.*(3) detected penicillin in the stimulated saliva of medicated animals. Long(4) observed changes in the bacterial flora of the mouth and inferred from this that penicillin was excreted into human saliva. Direct measurements of such excretion have not been reported.

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2. Struble, G. C. and Bellows, J. G., *J.A.M.A.*, 1944, v125, 685.
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TABLE I. Saliva Levels of Antibiotic Following Administration of Aureomycin or Terramycin Capsules to 32 Subjects.

Total dose (g)	Time of assay after last dose (hr)	No. of subjects	Range (μg/ml)	Mean (μg/ml)	Stand. error of mean
.5	2.5	31	<.06- .5	.13	.012
3.0	2.5	32	<.13-2.0	.42	.052
3.0	26.5	25	<.06- .5	.11	.026

TABLE II. Saliva Levels of Antibiotics Following Administration of Aureomycin or Terramycin Troches to 10 Subjects.

	1 min.*	10 min.	30 min.	1 hr	3 hr	24 hr†	27 hr	28 hr‡	30 hr
Range, μg/ml	80-640	20-80	10-40	5-20	.5-10	0	1.0-12.5	0-1.0	0-.25
Mean, μg/ml	260	51.5	20.5	11.2	4.4	0	4.98	.5	.1
S.E.	52.3	7.0	2.73	1.27	1.0	0	1.32	.03	.003

S.E.—Stand. error of the mean.
*—After first troche.
†—Second troche administered following this sample.
‡—Test made after lunch.

We found that following the ingestion of aureomycin or terramycin in capsule form, the saliva becomes inhibitory to *B. cereus*(5), presumably due to the salivary excretion of the antibiotics. In order to ascertain the source of the antibiotics in saliva we catheterized the parotid and submaxillary ducts of 4 subjects who had been medicated with aureomycin capsules. Both parotid and submaxillary saliva gave positive assays. It appeared that the major salivary glands excrete the antibiotic. In the present study, systemic and topical administrations of both drugs were compared by evaluating their saliva concentrations and their inhibitory effect on salivary microorganisms.

Method. Dosage and sampling. A. *Capsules.* Each subject took two 0.25 g capsules of antibiotic every 4 hours until a total of 3 g was consumed. Samples of blood and unstimulated saliva were collected 2½ hours after the first dose, 2½ hours after the last dose, and 24 hours later. In some cases a control sample of unstimulated saliva was obtained before medication. The serum and saliva samples were assayed for contents of antibiotic and the number of bacteria present in saliva was determined. B. *Troches.* Each subject allowed one 15 mg troche to dissolve in his mouth on each of 2 successive mornings. On the first day, saliva samples were collected 1 minute, 5 minutes, 10 minutes, 30 minutes, 1 hour, and 3 hours after the troche was completely dissolved. On the second day, samples of saliva were obtained 3 hours, 4 hours, 5 hours, and 6 hours after dissolution of the troche. A control sample of unstimulated saliva was collected before each medication. The specimens were used for determinations of antibiotic concentration and for bacterial counts.

Assays. A tube dilution method(6) testing inhibition of *B. cereus No. 5 var. mycoides*[†] was employed and all specimens assayed at two different dilutions. Aureomycin standard endpoints were accepted only when they occurred in the fourth or fifth tubes. All samples were kept under refrigeration until ready for incubation except for the short time necessary for dilution and inoculation. The data for assay readings reported in Tables I and II are calculated from the observed values without any correction for the error of the method. The latter has been shown to range from zero to +400%(6). For statistical handling of data, values below the range of a particular assay were counted as zero, though they may have amounted to as much as .25 μg/ml.

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[†] The original strain was kindly supplied by Mr. A. C. Dornbush of Lederle Laboratories Division.

TABLE III. Colony Counts ($\times 10^7$) of Viable Bacteria in Saliva Before and After Administration of Aureomycin or Terramycin Capsules to 12 Subjects.

		.5 g		3.0 g		24 hr later	
	Before	Test	Control	Test	Control	Test	Control
Range	3.6-65	3.6-13.4	4.2-33	.2-7.2	2.3-100	1.6-16.9	2.3-100
Mean	15.40	7.77	12.70	3.16	31.10	9.37	31.10
S.E.	2.57	.85	1.91	.70	2.73	1.60	2.73
t_1*		2.82 (1% Sig.)		4.59 (1% Sig.)		2.02 (5% Sig.)	
t_2†			2.51 (5% Sig.)		9.70 (1% Sig.)		6.90 (1% Sig.)

* Comparing estimates following medication with those before medication (test vs. before).
† Comparing estimates at same hour of different days (test vs. control).

Bacterial counts. Saliva specimens were diluted serially by tenths in tryptic digest broth and duplicate 0.1 ml amounts of the third, fourth, fifth, and sixth dilutions were plated out with 5% horse blood agar. The poured plates were incubated at 37°C for 24 hours, and the colonies were counted with the aid of a Quebec colony counter according to standard methods(7). Two sets of controls were employed in the series testing the effect of capsules (Table III). The customary pre-experimental count served as one reference; the other consisted of colony counts repeated at the hours of the test on another day, with the same subjects, but without medication.

Results and discussion. Assays following administration of capsules. (Table I). Twenty subjects were studied with aureomycin and 12 with terramycin. Among 88 saliva assays, 29 were negative, *i.e.*, their concentrations were below the range of the particular assay. Among the remainder no statistical difference appeared that would justify separate tabulation of the aureomycin and terramycin series. Despite the failure in 29 assays to obtain demonstrable levels of antibiotic in saliva, the average effect was positive. The mean level was significantly different from zero at all 3 instances and, according to the Student's "t"-test, the probability of a difference was 99% or better. The antibiotic concentrations in plasma ranged from 4-10 times as great as in saliva. If the salivary glands were able to concentrate the antibiotic, we should have found levels higher in saliva than in blood. This happened only twice among 120 parallel determinations. The mode of excretion, therefore, is unlike the excretory mechanism of the kidney. The ratio of concentration in saliva to the concentration in plasma seems comparable with the proportion described for penicillin excretion in human milk(8).

Assays following administration of troches. (Table II). Five test subjects were examined with each of the 2 antibiotics. Since no statistical difference between the series was observed, they were treated together. The concentration was very high originally, dropped steadily during the 3 test hours, and no antibiotic could be detected at the end of 24 hours. After another troche, the 3 hour levels were comparable to those of the first day, which fact reaffirms the absence of any traces of antibiotic after 24 hours. Following ingestion of food the antibiotics were still detectable in 9 out of 10 cases, while 2 hours later only 4 concentrations were assayable.

Inhibition following administration of capsules. (Table III). Six subjects were tested with each antibiotic and the colony counts tabulated together. Notwithstanding the wide variation in control counts, the reduction in viable salivary bacteria following medication appeared to be highly significant. It was noticeable 2½ hours after the first .5 g dose, it became very obvious after the full dosage, and in 7 out of 10 cases it persisted for 24 hours thereafter. Highest mean inhibition

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TABLE IV. Colony Counts ($\times 10^7$) of Viable Bacteria in Saliva Before and After Administration of Aureomycin or Terramycin Troches to 10 Subjects.

	Before	1 min.*	10 min.	30 min.	1 hr	3 hr	24 hr†	28 hr‡
Range	1.53-15.2	.21-4.8	.19-3.2	.04-2.3	.05-1.4	0-3.6	.13-8.8	0-1.1
Mean	5.19	1.44	1.17	1.01	.68	.97	2.28	.31
S.E.	1.43	.41	.34	.19	.08	.34	.86	1.29
% Red.	0	72.6	77.4	80.4	86.6	81.3	58.0	94.0
Signif. %		5	5	1	1	1	N.S.	5

S.E.—Stand. error of the mean.
*—After first troche.
†—Second troche administered following this sample.
‡—Test made after lunch.

amounted to 79.5% and 89.8% of the two mean base lines, respectively. The highest number of microorganisms per ml of saliva counted was 1 × 10^9, the lowest under medication was 2 × 10^6.

Inhibition following administration of troches (Table IV). Five subjects were tested with each antibiotic. Within the first minute after dissolution of an aureomycin or terramycin troche a reduction in colony counts became apparent. The difference between the mean test count and the mean pre-experimental control count was statistically significant. The individual deviations from the mean narrowed under medication. After some fluctuations, the estimates among 9 out of 10 subjects reached their lowest point 1 hour later. At this point the mean reduction amounted to 86.6%. Bacterial counts of saliva usually rise gradually between meals. The reductions observed in our tests occurred at periods when the bacterial population would normally be increasing. Feirer and Leonard(9) have shown that bacterial counts of saliva increase almost 300% over a fasting period of 7 hours and others(10-12) have found that the counts are lower after meals than before.

Inhibition following administration of two troches consecutively (Table V). One group of 8 subjects received one troche of aureomycin, and another group was given a troche of terramycin. One hour later the mean estimates of their salivary microorganisms were significantly reduced and comparable to those in the preceding series (Table IV). Immediately thereafter, all subjects received another like troche to test for "additive" action. Five minutes after dissolution of the second troche 15 of the 16 bacterial counts showed another significant decrease. The mean total reduction at this point was 97.1% and the mean number of organisms per ml of saliva was reduced from 67 million to fewer than 2 million. The count recorded as zero showed fewer than 10,000 microorganisms per ml. It is of practical interest that this high degree of inhibition persisted unchanged for the remaining hour of the test.

Summary. (1) Aureomycin and terramycin are excreted in saliva when administered by mouth in capsule form. (2) The salivary glands do not concentrate the material excreted from plasma. (3) On an average, salivary excretion lasts for 24 hours after capsule medication has been discontinued. (4) A 3 g dose reduces bacterial counts in saliva, and the inhibitory effect seems to last

TABLE V. Colony Counts ($\times 10^7$) of Viable Bacteria in Saliva Before and After Administration of Two Consecutive Troches of Aureomycin or Terramycin to 16 Subjects.

	Before	1 hr*	5 min.†	1 hr†
Range	.97-36.7	.12-1.75	0-1.39	.03-3.50
Mean	6.72	.824	.192	.184
S.E.	1.31	.158	.053	.032
% Red.	0	84.8	97.1	97.3
Signif.		1%	1%	N.S.

* After dissolution of first troche.
† After dissolution of second troche.

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for 24 hours. (5) One 15 mg troche decreases bacterial counts significantly for at least one hour. (6) A second troche administered one hour after the first adds considerably to the antibacterial effect.

We wish to express our appreciation to Miss Constance M. Marginot for her technical assistance; and to Mrs. Joan Keyser for the statistical handling of our data.

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Liver Storage of Vitamin B_{12} by the Rat.* (19139)

HAROLD E. SCHEID, MONA M. ANDREWS, AND B. S. SCHWEIGERT.
From the Division of Biochemistry and Nutrition, American Meat Institute Foundation, and Department of Biochemistry, University of Chicago, Ill.

Assays for vit. B_{12} by the use of rat and chick growth, or microbiological methods, are well known. Although available information (1,2) indicates that the amount of vit. B_{12} in the liver or other organs was lower for animals fed a vit. B_{12} deficient diet as compared to a supplemented diet, it was of interest to extend these studies and to determine the vit. B_{12} potency of the livers of rats fed graded amounts of vit. B_{12}. Further, the liver storage of the vitamin, when fed as the crystalline vitamin or as a food, could be evaluated as a method for determining the vit. B_{12} potency of foods and could be compared with data obtained by the microbiological and animal growth methods. These studies have been conducted, and data have also been obtained on the effect of the level of vit. B_{12} fed on the liver weight to body weight ratio.

Experimental and results. Weanling male rats of the Holtzman strain were fed a basal ration (corn-soybean oil meal-iodinated casein) without added vit. B_{12} as a depletion ration for a period of 2 weeks. The rats were then randomized into groups and fed either 0, 3, 6, 9, 15 or 24 μg of crystalline vit. B_{12} per kilo of ration or crude supplements of unknown vit. B_{12} potency for a period of 4 weeks. The ration and procedure used have been described previously(3). Seven rats were used in each group. After completion of the rat growth assay, all animals from selected groups were retained for studies on the storage of vit. B_{12} in the liver. The animals were sacrificed by decapitation, the livers removed and weighed. Two gram samples were homogenized in a Waring blendor with 50 ml of 0.1 M phosphate buffer, pH 6.8, for one minute, then autoclaved for 5 minutes at 15 lb pressure. After autoclaving, the samples were cooled, made to 100 ml volume, filtered and stored in the refrigerator. Aliquots of these samples were taken for microbiological assay according to the method previously described(4,5). The enzymatic digestion step was eliminated since investigations with short-term autoclaving of the samples as compared to subsequent enzyme

TABLE I. Vitamin B_{12} Content of Rat Livers As Assayed Using *Lactobacillus leichmannii.*

μg B_{12} added/kg ration	μg B_{12}/g liver*	μg B_{12}/liver	Liver wt (% of body wt)
0	.026 ± .005	.200	6.20
3	.030 ± .003	.306	6.49
6	.037 ± .011	.361	6.09
9	.047 ± .009	.474	5.77
15	.048 ± .006	.442	5.20
24	.066 ± .017	.621	5.00

* Mean and stand. dev.

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* We are indebted to Merck and Co. for supplying the vit. B_{12} used in these studies. Journal Paper No. 45, American Meat Institute Foundation.

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TABLE II. Vitamin B_{12} Activity of Liver With and Without Alkali Treatment.

Material	Test organism	Sample treatment Autoclaved pH 7, μg/g	 Autoclaved Alkali, μg/g
Beef liver	*L. leichmannii*	.77	.146
	Euglena gracilis	.74	0
Rat liver (B_{12} deficient diet)	*L. leichmannii*	.04	.031
	Euglena gracilis	.02	0
Rat liver (B_{12} supplemented diet)*	*L. leichmannii*	.068	.057
	Euglena gracilis	.054	0

* 24 μg of vit. B_{12}/kg of diet.

treatment gave identical results on three assays. Duplicate analyses were made for each liver and *Lactobacillus leichmannii 327* (ATCC 7831) was used as the test organism.

The vit. B_{12} potency of the liver (0.066 ± .017 μg/g) for the group fed an optimum level of vit. B_{12} was considerably higher than that for the basal group (0.026 ± .005 μg/g) as shown in Table I. In a subsequent experiment, livers from rats fed the basal diet and basal diet plus 24 μg B_{12} per kg diet were analyzed for vit. B_{12} in a similar manner. In this experiment, the basal group averaged 0.046 μg/g of vit. B_{12} per g of liver, and the supplemented group averaged 0.108 μg. These values for the B_{12} potency of rat livers are approximately 1/10 to 1/20 those observed for beef liver(5).

Since values for the vit. B_{12} potency of rat liver appeared low compared to that of beef liver, it was of interest to determine how much of the B_{12} potency in these samples may be attributed to desoxyribosides. Hoffman, *et al.*(6) showed that vit. B_{12} was destroyed by heating with 0.2 N NaOH at 100°C for 30 minutes and that under these conditions the vit. B_{12} activity of the desoxyribosides of thymine, guanine, and hypoxanthine was not affected. They also found that about 97% of the total microbiological activity of liver extract disappeared after alkali treatment. Rat liver was subjected to a similar treatment: Two g samples were homogenized in 40 ml 0.1 N NaOH and autoclaved at 15 lb pressure for 30 minutes. The samples were neutralized with 2 N HCl, made to 100 ml volume and filtered. Aliquots were then taken for microbiological analyses. Rat liver homogenates treated with alkali were found to retain an appreciable amount of vit. B_{12} activity for *L. leichmannii* (Table II). When rat liver plus crystalline vit. B_{12} was so treated, the added B_{12} was destroyed, about 80% of the vit. B_{12} potency of beef liver was destroyed, but the vit. B_{12} activity of rat liver was retained. It is known that *L. leichmannii* responds to thymidine and desoxyribosides (7-9), and since rat liver contains alkali stable substances with vit. B_{12} activity, it is likely that the organism measured desoxyriboside activity after alkali treatment. Vit. B_{12} complexes that may be present in liver that are stable to alkali could also explain these findings.

This problem was further investigated by the use of the algal flagellate *Euglena gracilis.* Hutner, *et al.*(10) have shown that thymidine does not replace the vit. B_{12} requirement of this organism. The medium used in these studies was that of Robbins, *et al.*(11) with the exception of NH_4VO_3 which was not included. The method of assay was essentially that of Hutner,† *et al.*(10). The organism was cultured on an agar slant containing

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0.5% peptone, 0.1% liver extract, 0.2% sodium acetate and 1.5% agar. The inoculum was prepared in the same medium (pH 7) without the agar. An assay period of 6 days was used, and the amount of growth determined by measuring the turbidity of the cultures with the use of a Coleman Junior Spectrophotometer.

Fresh rat and beef liver homogenates with and without alkali treatment were assayed with *Euglena*. The results indicated that the values for livers from vit. B_{12}-deficient rats were consistently lower in vit. B_{12} than those for livers from rats fed an optimum level of vit. B_{12}. The values obtained by the vit. B_{12} *Euglena* method of assay for rat or beef liver treated with alkali were negligible. The results for fresh beef and rat liver with this method of assay compared very well with the values found by the *L. leichmannii* assay (Table II).

These studies indicate that application of the procedure of alkali treatment to low potency samples, that retain a large portion of the vit. B_{12} activity after alkali treatment for *L. leichmannii*, may not provide information that can be reliably interpreted. Other studies(12) have shown that the desoxyribonucleic acid level of livers from vit. B_{12}-supplemented rats is 10-20% higher than for deficient rats. Thus, the increases observed in the vit. B_{12} activity of rat liver when the amount fed was increased can be only partly explained by increased desoxyriboside activity. Although the data obtained after alkali treatment are not definitive, the results for *L. leichmannii* and *Euglena*, as well as the rat assay(1), show that the vit. B_{12} stored in the liver is related to the vit. B_{12} intake.

It is of interest that the liver weight was greater per unit of body weight for animals fed the low levels of crystalline vit. B_{12}. Among the deficient animals, the liver weight was found to be 6.2% of the body weight, while the percentage for the B_{12}-supplemented group was 5.0 (Table I). These data are in agreement with those observed in another experiment in which the percentage of body weight that was liver weight was 6.6 for animals fed the basal diet and 5.2 for animals fed the basal diet plus 24 μg vit. B_{12} per kg.‡ The percentage for animals fed the supplemented diet with the food intake restricted to that of the basal group was 5.0.

By the use of the liver storage method of assay, values for fresh beef round, pork ham, lamb leg, kidney, liver, and injectable liver extract have been determined. In the cases of pork and lamb, the data for vit. B_{12} are in close agreement with those for similar samples studied earlier using the microbiological technic. The values for the remainder of the crude materials tend to be somewhat higher. Details of these studies will be reported at a future date.

Summary. The storage of vit. B_{12} in rat liver was investigated after feeding rats graded levels of crystalline vit. B_{12} or of crude supplements. The vit. B_{12} potency was determined with *L. leichmannii* after short-term autoclaving of the liver homogenates at pH 7.0. The vit. B_{12} potency was shown to increase as the level fed was increased. The values were only 1/10 to 1/20 those for beef liver, however, and the vit. B_{12} potency of rat liver was not reduced appreciably by alkali treatment. Studies on the desoxyribonucleic acid levels in rat livers and studies with *Euglena gracilis* assays for vit. B_{12} indicated that the vit. B_{12} activity remaining, as determined with *L. leichmannii* after alkali treatment, may be due to the presence of alkali stable forms of vit. B_{12} as well as to desoxyribosides. The liver weight was shown to be greater per unit of body weight for the rats fed the vit. B_{12}-deficient diet as compared to the optimum diet.

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† We are indebted to Dr. S. H. Hutner for providing a culture of *Euglena gracilis*.

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Pressure Measurements in Perfused Segments of Small Blood Vessels.[*] (19140)

Victor M. Emmel and Durwood J. Smith.[†]

From the Departments of Anatomy and Medicine, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

A plethysmographic method has recently been described(1,2) for evaluating *in vitro* the responsiveness of blood vessels during perfusion with various agents. The application of this technic to very small vascular segments (5 to 10 mm long and 1 mm diameter, such as obtained from dog mesentery) was not entirely satisfactory because of the difficulty in measuring the small volume changes involved. However, by measuring the rather large pressure differences which even these small specimens can develop, it is possible to extend the perfusion technic to vessels of any size which can be cannulated. The measurement of pressure responses has a further advantage in the fact that the pressure which a vascular segment can develop is independent of its length; whereas the volume change which it can undergo is, of course, directly proportional to its length. Hence, pressure responses provide a more satisfactory basis for quantitative comparisons between various specimens.

Materials and methods. The essential feature of this technic is that the specimen is tied between cannulae and placed within a fluid-filled chamber to which is attached an electrical pressure transducer of very small displacement (Fig. 1). The standard-taper bushing adapters were shortened to correspond to the medium length outer members into which they fit. The cannulae are appropriately sized hypodermic needles with ends squared and grooved. Perfusate flows by gravity at a constant pressure of about 35 mm Hg. An increase of vascular tone under these circumstances can produce no change in pressure within the vessel, but does produce a "negative" pressure in the external chamber. Since no significant volume change can occur, this response represents an essentially isometric contraction of the smooth muscle in the vascular wall. If the diameter of the vessel and thickness of the muscular portion of the wall are known, the muscular tension per unit cross-sectional area can be calculated (3). A Hathaway blood pressure recording system is currently employed with this apparatus, but any pressure transducer of small displacement together with a suitable recording device would be equally satisfactory. Temperature is maintained at 37.5°C. Test substances are injected into the perfusate through a T-tube in the perfusion line. In order to define the treatment to which the

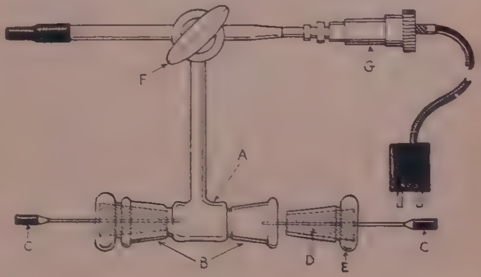

FIG. 1. Diagram of perfusion apparatus. Perfusion chamber (A) is formed by uniting 2 standard taper 14/20 ground glass joints (B). Cannulae (C) are inserted through rubber stoppers (D) placed within standard taper 14/35—10 bushing-type adapters. One cannula should be long enough to extend completely through the perfusion chamber. Silicone lubricant facilitates adjustment of cannulae. The T-stopcock (F) permits the chamber to be filled and placed in communication with an electrical pressure transducer (G).

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[*] An abstract based on part of this work appears in *Anat. Rec.*, 1951, v109, 30.

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specimen is subjected it is necessary to know both the intravascular concentration and the time of arrival of the test substance. These depend essentially on the perfusion rate and the dimensions of the perfusion line between the injection site and the tip of the inflow cannula. They can, therefore, be evaluated by injecting a standard volume of a dye (0.05 ml of 1% congo red) and then collecting and colorimetrically analyzing closely spaced samples of perfusate issuing from the inflow cannula. The system can thus be calibrated for various perfusion rates. Vasoactive substances to be tested are so diluted that test doses of 0.05 ml will contain the desired amount of the test substance.

Results and comments. This method can readily be applied to large vessels; but it is particularly useful in extending perfusion studies to vessels of very small caliber. It has thus far been applied to vessels as small as the carotid and renal arteries of the rat and arterial biopsy specimens obtained from the mesentery of the dog. Fig. 2 represents a typical result obtained from a dog's mesenteric artery perfused with Tyrode's solution and stimulated with epinephrine. It is apparent that the maximal intravascular concentration of epinephrine (0.8 γ/ml) has already traversed the vessel before the vasoconstrictor response begins. Increasing the dose above this value (up to 4.0 γ/ml) increases the duration but does not materially increase the height of the response. With some specimens similarly stimulated, maximal pressures approaching 300 mm Hg have been recorded. By using successively smaller doses it was determined that the threshold for response of this specimen was with an injected dose of 0.05 γ (corresponding to a maximal intravascular concentration of 0.04 γ/ml). Returning to Fig. 2 it can be determined that the interval between the onset of contraction and the time at which the concentration curve had reached a value of 0.04 γ/ml is 3.4 seconds. This interval is tentatively interpreted as the "latent period" for chemical stimulation. Using various test doses giving maximal intravascular concentrations of from 0.04 to 4.0 γ/ml the latent period was found in 20 determinations to be 3.7 ± .47 seconds. The factors which influence and contribute to this latent period are currently under investigation and will be considered in a subsequent report. This technic is also being used in a study of biopsy specimens from animals with experimental vascular disease.

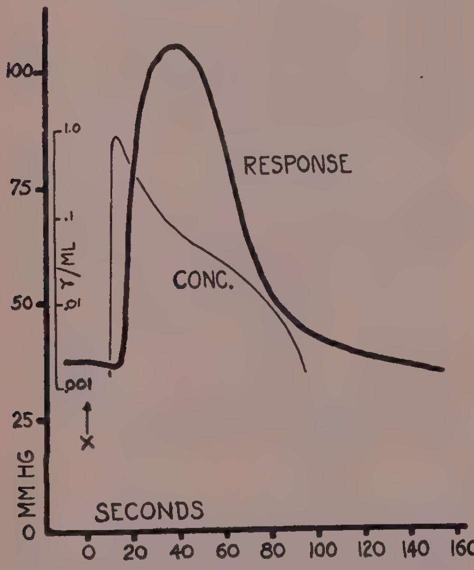

FIG. 2. Response of arterial segment (diameter 1 mm, length 7 mm) from mesentery of a dog. Perfused at a rate of 7.2 ml per minute with oxygenated Tyrode's solution. At X, 1.0 γ epinephrine in 0.05 ml injected into perfusion line. Concentration of epinephrine reaching specimen is shown by light line. Heavy line represents pressure response.

Summary. A method is described for recording *in vitro* pressure responses from perfused segments of small blood vessels. The method permits accurate evaluation of the relationships between dose and response. For arterial biopsy specimens from the dog's mesentery, maximal pressure responses of 200 to 300 mm Hg are obtained with test doses of epinephrine at an intravascular concentration of about 1.0 γ/ml. The threshold for response is about 0.04 γ/ml. The latent period for chemical stimulation of these specimens is approximately 3 to 4 seconds.

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Biological Activity of a Derivative of the Citrovorum Factor.* (19141)

G. E. Cartwright, M. M. Wintrobe, H. P. Broquist, and T. H. Jukes.

From the Department of Internal Medicine, University of Utah, College of Medicine, Salt Lake City, Utah and from the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

In 1948 Sauberlich and Baumann(1) described a factor (CF) present in liver which is necessary for the growth of *Leuconostoc citrovorum* 8081. The synthesis of a substance, "leucovorin"(2,3), "folinic acid SF" (4,5), 5-formyl-5,6,7,8-tetrahydro pteroylglutamic acid, with the biological properties of CF has recently been described. Leucovorin, unlike synthetic pteroylglutamic acid (PGA), has been shown to be active in reversing the growth inhibiting effects of Aminopterin (4-amino PGA) on *L. citrovorum*(6-8), mice(6), rats(9), chick embryos(10), and mouse leukemia(11). Studies in patients with Addisonian pernicious anemia indicate that leucovorin is active when given parenterally in amounts of 0.75 to 3 mg daily for 10 days (12-16). In infants with megaloblastic anemia this factor has been found to be active in amounts as small as 75 μg per day when given parenterally(17). Following the administration of PGA to man and rats, CF is excreted in increased amounts in the urine(18). Weight for weight CF appears to be more effective than PGA in converting megaloblasts to normoblasts in bone marrow cultures(19). These data, in addition to the observations that the conversion of PGA to CF by rat liver slices is inhibited by the administration of Aminopterin and that CF is capable of preventing this inhibition(9,20), have led to the conclusion that in the body limited amounts of PGA may be converted to CF. It has also been suggested that ascorbic acid and vit. B_{12} are concerned in this conversion of PGA to the biologically more active CF(20,21).

When CF is allowed to stand at room temperature in dilute acid it loses its activity for *L. citrovorum* but its activity for *S. faecalis* is retained(2,6). Because of the possibility that the hydrochloric-acid-treated leucovorin may be similar to the product which occurs when naturally-occurring CF is exposed to the normal action of gastric juice, it was thought to be of interest to study the bio-

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TABLE I. Comparative Microbiological Activity of Citrovorum Factor, Acid-Treated Leucovorin and Pteroylglutamic Acid.

Compound tested	*Leuconostoc citrovorum* Microbiological activity expressed as %	*Streptococcus faecalis*
Pteroylglutamic acid	0.001	100
Acid-treated citrovorum factor	2.0	53
Citrovorum factor	100	50

TABLE II. Effect of Citrovorum Factor (CF), Acid-Treated Citrovorum Factor (ATCF) and Pteroylglutamic Acid (PGA) on Toxicity of 4-Aminopteroyl Glutamic Acid for Mice. Supplement injected 3x weekly.

Group No.	4-NH_2 PGA	CF	ATCF	PGA	1 day	4 days	5 days
1281	—	—	—	—	20.2 (10)	22.2 (10)	23.1 (10)
82	10 γ	—	—	—	20.5 (10)	17.9 (10)	16.8 (8)
83	10 γ	10 γ	—	—	20.7 (10)	21.3 (10)	21.6 (10)
84	10 γ	—	30 γ	—	20.6 (10)	20.5 (10)	19.9 (10)
85	10 γ	—	100 γ	—	20.6 (10)	21.8 (10)	22.2 (10)
87	10 γ	—	—	100 γ	20.4 (10)	19.0 (10)	17.5 (10)

	8 days	10 days	12 days	15 days
1281	24.7 (10)	25.2 (10)	25.8 (10)	26.8 (10)
82	—	—	—	—
83	22.0 (10)	21.8 (10)	21.1 (10)	20.7 (9)
84	19.5 (6)	19.0 (4)	22.5 (2)	25.0 (1)
85	23.6 (10)	23.6 (10)	23.2 (10)	24.5 (9)
87	16.3 (3)	18.5 (2)	17.0 (2)	16.0 (1)

logical activity of this product (ATL) in bacteria and mice and in patients with pernicious anemia in relapse.

Experiments. Leucovorin, 5-formyl-5,6,7,8-tetrahydro-pteroylglutamic acid(2,3) was synthesized by Drs. B. Roth, M. E. Hultquist, and J. M. Smith, Jr., Calco Chemical Division, American Cyanamid Company. A solution for clinical use was prepared by dissolving the calcium salt in water and adjusting the concentration to 3 mg per ml calculated as the anhydrous free acid, 0.15 mg of which corresponds to 1,000,000 "units"(22). The solution was placed in 1 ml ampules and sterilized by autoclaving. "Acid treated leucovorin" (ATL) was prepared by allowing leucovorin solution (4 mg per ml) to stand at pH 2 for 24 hours at 25°C. The solution was then neutralized, brought to a concentration equivalent to 3 mg per ml of original leucovorin and sterilized by autoclaving. *Leucovorin citrovorum* 8081 and *Streptococcus faecalis* 8043 were used in this study. The assay technic with *L. citrovorum* was that described by Sauberlich(17) with leucovorin as a standard. The microbiological assay procedure with *S. faecalis* followed that of Flynn *et al.*(23) with synthetic pteroylglutamic acid (PGA) as a standard. In the experiments with mice the procedure of Franklin *et al.*(24) was used. Carworth Farms CF-1 strain white mice weighing about 20 g were placed on a purified diet without PGA and containing 1% succinylsulfathiazole. Three patients with Addisonian pernicious anemia in relapse were studied. The patients were hospitalized and during the assay periods, liver, meat, meat products, milk and poultry were excluded from the diet. Bread, cereals, sugar, fat, vegetables, and fruits were permitted in the amounts desired.

Results. The response of *L. citrovorum* and *S. faecalis* to pteroylglutamic acid, leucovorin

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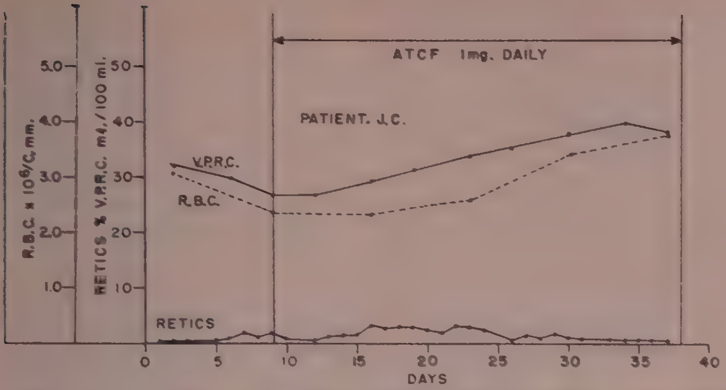

FIG. 1. Response of a patient (J. C.) with pernicious anemia in relapse to the intramuscular administration of 1 mg of acid-treated leucovorin (ACTF) daily for 29 days.

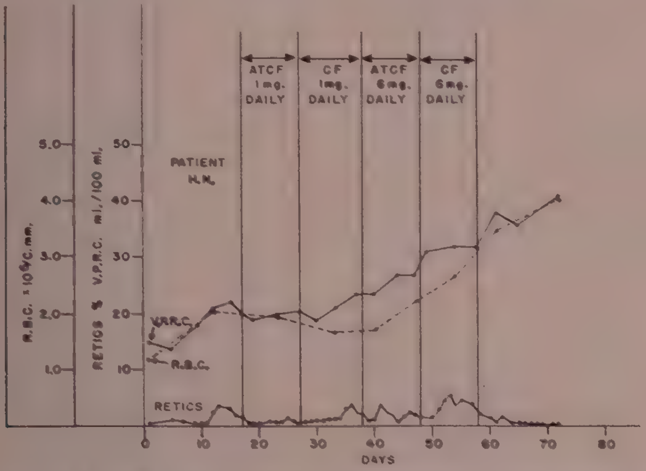

FIG. 2. Response of a patient (H. N.) with pernicious anemia in relapse to the intramuscular administration of acid-treated leucovorin (ATCF), and leucovorin (CF).

and acid-treated leucovorin (ATL) are shown in Table I. About 100,000 times more PGA was required than leucovorin for equivalent growth of *L. citrovorum*. As reported elsewhere(25) PGA was about twice as active as leucovorin for *S. faecalis*. Exposure of leucovorin solutions to pH 2 at 25°C resulted in inactivation of leucovorin activity, while the *S. faecalis* activity was unaltered.

The ability of leucovorin, ATL, and PGA to reverse the toxic effects of 4-amino-pteroylglutamic acid (Aminopterin) in mice is summarized in Table II. Reversal of the lethal effect of 10 μg of Aminopterin (90% survival) was obtained with 10 μg of CF. A somewhat larger amount of leucovorin was noted previously to be required to produce a gain in weight equal to that of a control group not receiving Aminopterin(2). PGA was almost completely ineffective in reversing Aminopterin toxicity in mice. Under the conditions of this experiment ATL was about one-tenth as active as leucovorin in reversing the effect of the antagonist.

The hematologic responses of the 3 patients with pernicious anemia in relapse to various doses of intramuscularly administered ATL and leucovorin are shown in Fig. 1-3. The

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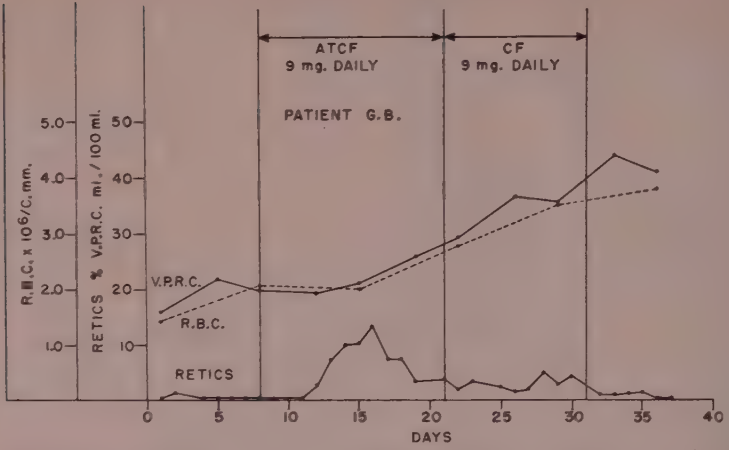

FIG. 3. Response of a patient (G. B.) with pernicious anemia in relapse to the intramuscular administration of acid-treated leucovorin (ATCF), and leucovorin (CF).

administration of 1 mg of ATL daily for 29 days was associated with an increase in the volume of packed red cells (VPRC) from 27 to 40 ml/100 ml and a rise in the red cell count (RBC) from 2.37 million per cmm to 3.80 in this period of time, although a significant reticulocyte response was not observed (Fig. 1). The administration of 1 mg ATL to a second patient (Fig. 2) for 10 days was without apparent effect on the RBC, VPRC or reticulocytes. However, the subsequent administration of 1 mg of leucovorin daily for 10 days was associated with a slight increase in reticulocytes to 3.8% and a significant rise in the VPRC. Six mg of ATL was then given daily for 10 days and the RBC and VPRC continued to increase. When 6 mg of leucovorin was given daily for 10 days a reticulocyte rise to a peak of 5.6% took place and the increase in RBC and VPRC continued. The third patient (C.B.), (Fig. 3) was given 9 mg of ATL daily for 13 days. This resulted in a rise in reticulocytes to 13.4% and a significant increase in RBC and VPRC. The administration of 9 mg of leucovorin daily for 10 days following this was associated with a very slight secondary reticulocytosis of 5%.

Discussion. In confirmation of earlier studies it is apparent that, although *S. faecalis* can utilize either PGA, CF or ATL for its requirement for folic acid, *L. citrovorum* can utilize only CF. The studies with mice suggest that in the presence of Aminopterin the mouse is able to convert the acid-treated product to CF to a limited extent. The results in patients with pernicious anemia in relapse indicate that the acid-treated product is less active than leucovorin. This was apparent at levels of 1, 6 and 9 mg. Evidence published to date(12-16) as well as the observations reported here, indicate that amounts of leucovorin greater than 1.0 mg are required to produce a satisfactory hemopoietic response in cases of pernicious anemia. Davidson and Girdwood(15) failed to obtain maximal reticulocyte responses after the administration of a single injection of 12 mg. Meyer *et al.*(13) obtained the expected reticulocyte maximum in only one of 4 patients given 3 mg intramuscularly each day for 10 days. Ellison *et al.*(14) found that 4 patients treated with 0.75 to 1.5 mg of leucovorin daily developed maximal red cell rises at the end of 3 weeks, although the reticulocyte responses were only about one-half that expected.

It seems, therefore, that the therapeutic doses of leucovorin and PGA are of the same order since the oral administration of PGA in amounts of 1.0 to 2.5 mg per day results in satisfactory although sub-optimal responses in patients with pernicious anemia(25). This would seem to indicate that the conversion of PGA to CF is not greatly disturbed in patients with pernicious anemia. Such a finding somewhat contradicts the hypothesis that vit. B_{12} is concerned in the conversion of PGA to CF (21) for, if such were true, CF should be considerably more active than PGA on a weight for weight basis.

Summary. Exposure of citrovorum factor (leucovorin) solutions to pH 2 for 24 hours at 25°C resulted in inactivation of *L. citrovorum* activity but produced no alteration in the activity for *S. faecalis*. The acid-treated leucovorin was about one-tenth as active as leucovorin in reversing Aminopterin toxicity in mice. When administered to patients with pernicious anemia in relapse the acid-treated leucovorin was less active than leucovorin.

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Effect of Hypoxemia on Edema Formation in Perfused Isolated Rat Hind Limb.* (19142)

Edith L. Di Pasquale and Alfred A. Schiller.

From the Department of Physiology, University of Illinois College of Medicine, Chicago.

The effect of hypoxemia on capillary function has been the object of numerous investigations since Landis(1) noted that complete anoxemia caused a fourfold increase in capillary filtration rate in the frog mesentery. Maurer(2,3) used the rate of flow and protein content of the cervical lymph in dogs to indicate a change in permeability of the capillaries during respiratory hypoxemia. In all cases lymph flow increased steadily with progressing hypoxemia to as low as 7 volumes % oxygen content. However, this change cannot be attributed to hypoxemia alone when considering that many other factors affect the overall flow of lymph. Warren and Drinker(4) studied the lung lymph flow in the same manner using a level of hypoxemia as low at 4.4 vol. %, and found that lung lymph flow consistently increased. Further indirect evidence that graded hypoxemia causes graded increases in capillary permeability was reported by Pochin (5), who occluded the blood vessels at the base of the rabbit's ear for 2 to 24 hours and noted an increase in the thickness of the ear in proportion to the time of occlusion. Hopps and Lewis(6) found that anoxemia did not facilitate the passage of antibody globulin nor of T-1824 through the vascular epithelium of the guinea pig. McMichael and Morris(7) as well as Henry *et al.*(8) utilized the procedure of venous congestion of the upper extremities of human beings. The former authors found no increase in swelling of the arm at 9.5 vol. % oxygen content and the latter no change in calculated loss of protein from the blood of the congested arm at 60 to 70% oxygen saturation of the blood. However, at 15 to 20% saturation or 4 to 6 vol. % oxygen content a significant loss of protein occurs(9). Stead and Warren(10) noted that in patients with chronic emphysema in whom the blood was 50 to 60% saturated, no signs of protein "leakage" were present. Perfusion technics in the frog were used by Danielli(11) and Saslow(12) with different interpretations

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* Aided by a grant from the National Heart Institute of the National Institutes of Health, U. S. Public Health Service.

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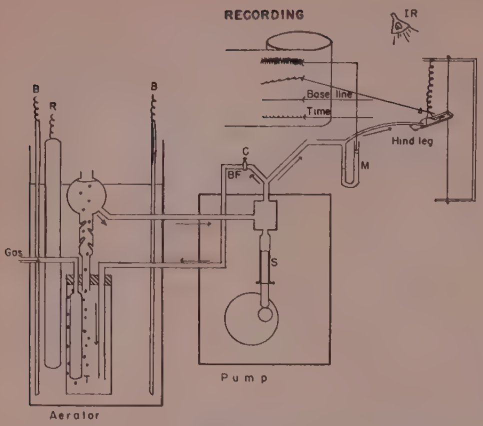

FIG. 1. Perfusion apparatus. T, scintered glass tube; B, heating blades; R, thermoregulator; C, screw clamp; BF, backflow tube; S, syringe; M, mercury manometer; IR, infra red lamp. Description in text.

of the effectiveness of using red blood cells in the perfusion medium to prevent edema. Saslow attributes the beneficial effect of red blood cells to their oxygen carrying capacity whereas Danielli concluded that in addition to the availability of oxygen the presence of platelets in blood perfusates was also necessary to clog the "pores" in the capillary endothelium and thereby prevent edema. It is apparent from the above that a consensus is lacking concerning the effect of hypoxemia on capillary permeability, especially within the physiological range.

In the following investigation a modification of the method of Hyman(13) was chosen to study quantitatively the effect of hypoxemia on capillary function. It consists of perfusing isolated rat hind limbs and determining the rate of edema formation by recording continuously the weight of the perfused leg.

Methods. The perfusion apparatus (Fig. 1) contains the aerators designed on the principle of the Clark *et al.*(14) oxygenator. A methylpolysiloxane resin, Dow Corning Antifoam A, is used to coat the inside surface of the aerators. A scintered glass aerating tube disperses the gas into fine bubbles and also serves as a stirring mechanism. The reservoirs are in a water bath maintained at 41°C by a thermoregulator operating 2 heating blades and a stirrer to ensure the delivery of fluid to the hind limb at 37°C. Separate reservoirs are used for the hypoxic and for the oxygenated perfusion fluids. Variations in oxygen content are achieved by bubbling 100% oxygen for at least 1 hour, or 100% nitrogen that has been bubbled through alkaline pyrogallol to remove any oxygen(15). Three-way stopcocks connecting both reservoirs with the perfusion pump permit a rapid change of fluids. The perfusion pump consists of an automatic pipetting machine to which a 3 cc siliconed syringe is attached with the barrel fixed at the Luer-Lok tip and the plunger attached to a cam which pushes or pulls the plunger as it rotates eccentrically. The continually rotating cam provides a pulsatile flow of fluid at a rate of 40 strokes per minute. On the downstroke of the plunger, fluid enters the syringe from the aerators. On the upstroke fluid is pumped through a valve assembly into a Y-tube. One branch of the Y, a backflow tube, conveys fluid back to the aerator and the other leads to the cannula into the hind limb. Since the greater portion of the output of the syringe is returned to the aerator the turnover of fluid therein is very rapid and stirring of the red blood cells is

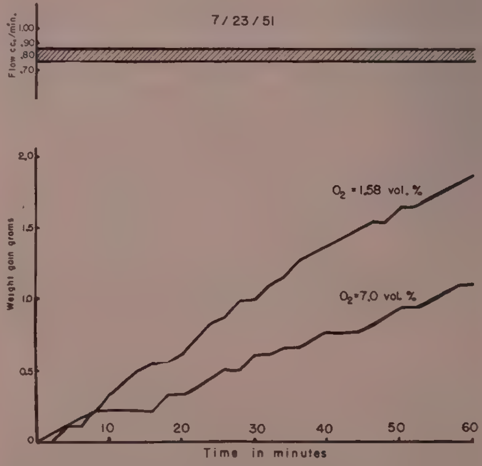

FIG. 2. Representative data relating weight gain, blood flow and oxygen content of perfusion fluid as a function of time.

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TABLE I. Summary of Data Relating Edema Formation with Oxygen Content and Flow Rate of the Perfusion Fluid.

Time, min	No. of expts.	Perfusion fluid, O_2 vol %	Avg mean press., mm Hg	Avg flow, cc/min	Avg wt gain, g/min/100 g
0-20	6	.88-2.60	41	.54	.142
	5	5.50-9.40	67	.60	.100
21-40	6	.88-2.60	31	.52	.086
	5	5.50-9.40	60	.60	.046
41-60	6	.88-2.60	32	.52	.093
	5	5.50-9.40	67	.60	.037

more effectively achieved. A screw clamp on the backflow tube could be adjusted to permit any desired flow through the cannula. A mercury manometer is placed in the perfusion system at the attachment of the cannula for the continuous recording of mean perfusion pressure on a slowly moving kymograph. The isolated preparation is placed on an aluminum pan suspended from a sensitive spring. At the junction of the spring and the pan one end of a heart lever is inserted so that a downward displacement of the weight pan is magnified 14 times by the mechanical advantage of the lever. The weight pan is allowed to move freely in a vertical plane along a guide wire. The pan is tilted so venous blood from the limb falls off the pan causing an instantaneous drop in weight and giving the weight tracing a serrated appearance. The number of drops falling off per minute is used as a measure of flow. At the beginning of each experiment the spring is calibrated by adding one gram weights and noting the vertical displacement of the lever, usually about 6 mm per gram. The slope of edema formation during a given period of time is calculated by the least squares method, expressing the slope in g/min./100 g of tissue.

Male albino rats weighing 250 to 350 g are anesthetized with ether and the right hind leg is cannulated with a flexible, fine polyethylene cannula in the iliac artery at the bifurcation of the aorta. The limb is dissected from the body at the hip, tying off all of the large vessels that are cut except the vena cava through which most of the outflowing blood passes. The perfusion is started immediately after the cannulation is complete to avoid stagnation of blood in the leg. The isolated limb is weighed on a beam balance before and after perfusion. An infra red lamp placed about 1½ feet from the leg prevents cooling of the exposed tissues. The perfusion fluids used consist of 20% washed dog red blood cells in 0.33% ash free gelatin in Kreb Ringer solution. The low colloidal content of these perfusion fluids is necessary to obtain a steady rate of edema formation within an experimental period of one to two hours. The pH of the fluids is adjusted to 7.4 with Na_2HPO_4 and is measured with a Beckmann glass electrode pH meter. Hemoglobin is determined by the Sahli method and the oxygen content by the Roughton and Scholander microgasometric syringe analyzer method(16).

Results. In the early experiments perfusions were carried out with hypoxic or with oxygenated blood at a constant mean perfusion pressure of 90 mm Hg. Under these conditions the flow during the hypoxemic perfusion was several times greater than during oxygenated perfusion in the same leg, thereby tending to compensate for the reduced oxygen content of the nitrogenated blood. Therefore, it was desirable to maintain flow constant in order to make manifest any changes due to hypoxemia *per se*. Consequently, in the following experiments the standard or reference flow was determined for each hind limb by perfusing it with oxygenated blood at 90 mm Hg mean perfusion pressure and noting the flow under these conditions. During the subsequent experimental period of one hour the flow was maintained at that reference level. Eleven perfusions were made; 6 with hypoxic blood (0.88 to 2.60 vol. %) and 5 with oxygenated blood (5.50 to 9.40 vol. %). Usually one hind leg was perfused with hypoxic blood

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16. Roughton, F. J. W., and Scholander, P. F., *J. Biol. Chem.*, 1943, v148, 541.

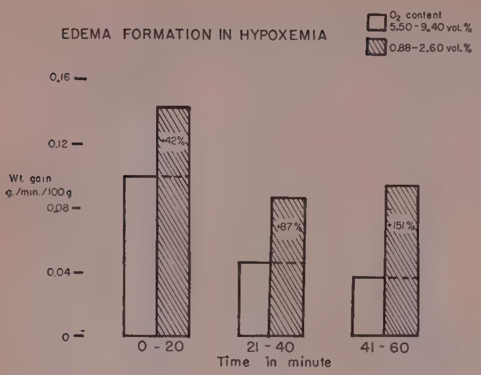

FIG. 3. Description in text.

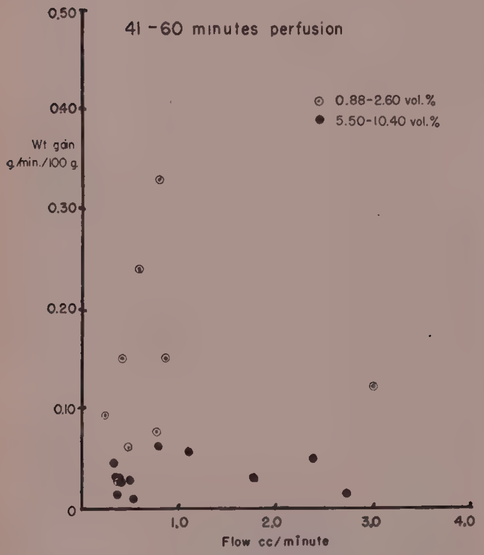

FIG. 4. Relationship between edema formation and flow.

and another with an oxygenated portion of the same blood for one hour. The slope of edema formation was calculated for the three consecutive 20-minute periods in each experiment. The protocols of 2 typical perfusions are shown in Fig. 2. The hypoxemic leg (1.58 vol. %) gained 1.87 g in one hour and the leg with oxygenated blood (7 vol. %) gained 1.1 g. In both cases the flow was maintained within a constant range of 0.75 to 0.85 cc/min. The results of 11 such experiments are summarized in Table I.

When the final column of rate of edema formation in g/min./100 g of tissue is plotted on a bar graph (Fig. 3) the effect of time and of oxygen content is more readily seen. With adequate oxygenation (solid bars) the edema rate is greatest in the first 20 minutes of perfusion and falls progressively during the rest of the hour. During hypoxemia, however, (striped column) the edema rate is 42% greater than the oxygenated values in the first 20 minutes, 87% greater in the next 20 minutes, and 151% higher from 41 to 60 minutes. This indicates that as anoxia of the tissues progresses, edema formation becomes more pronounced.

In Fig. 4 the blood flow is plotted against edema rate in 21 experiments in the last 20-minute period when the hypoxic changes were the most severe. During hypoxemia (dotted circles) the edema rate was approximately proportional to flow, whereas when the blood was fully oxygenated (solid circles) the edema rate was not altered significantly by flow.

Discussion. This preliminary work indicates that oxygen content above 5 vol. % plays no significant role in altering the rate of edema formation in the isolated rat hind limb perfused with blood low in colloidal content. At lower oxygen values an increased porosity of the capillaries is suggested since with increasing flow the rate of edema formation also increases. This was not demonstrated in the adequately oxygenated preparations.

We are in agreement with the conclusions of Henry(9) and Maurer(2,3) who state that the blood oxygen must fall below 4 to 6 vol. % or 15 to 20% saturation before a permeability change is noted. It would appear that the oxygen level at which a change in capillary function is detected is incompatible with life so that generalized hypoxemia can hardly be considered as an etiological factor in edema formation in chronic pathological states.

Summary. (1) A pulsatile perfusion system is described in which temperature, blood flow and mean pressure are controlled, and in which red blood cell solutions may be satisfactorily perfused through isolated limbs and organs. (2) In hind limbs perfused with blood of low colloidal content, 5.5 to 9.4 vol. % oxygen content were adequate in main-

taining capillary function since a minimal rate of edema formation was observed which did not vary appreciably with flow. (3) Hind limbs perfused with 0.88 to 2.60 vol. % oxygen exhibited augmented edema formation which increased with increased flow. The critical hypoxemia level affecting capillary permeability is believed to lie between 2.6 and 5.5 vol. % oxygen content for the experimental conditions described.

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Utilization of Ammonium Nitrogen for Growth and Toxin Production by a Strain of *Corynebacterium diphtheriae*. (19143)

RUTH M. DREW. (Introduced by W. M. Hale.)

From the Division of Bacteriology and Virology, Brookhaven National Laboratory, Upton, L. I., N. Y.

In general it has been believed that *Corynebacterium diphtheriae* utilized only nitrogen from organic materials. However, it has been shown by Drew and Mueller(1) that the Toronto strain of Park-Williams no. 8 readily utilizes ammonium nitrogen in addition to that from organic substances. On chemically defined medium it was found that in the presence of optimal concentrations of amino acids growth was poor without added ammonium sulfate or ammonium chloride. The fact that glutamic acid was an essential amino acid for the test strain and that the addition of ammonium salt resulted in greater growth suggested that perhaps ammonia was being utilized to convert glutamic acid to glutamine. Hac, Snell and Williams(2) and McIlwain(3) have demonstrated that certain microorganisms readily perform the conversion of glutamic acid to glutamine when ammonia is available. The possible role of ammonia in the conversion of glutamic acid to glutamine by the Toronto strain has therefore been investigated.

Quantitative studies involving the utilization of ammonium nitrogen for the growth of the Toronto strain of Park-Williams no. 8 are to be reported. It will be shown that glutamine in high concentrations can substitute for glutamic acid and ammonium sulfate for the growth of the test strain. Glutamine plus ammonium ion has a greater additive effect than glutamine alone, indicating that ammonia is used in some other way, or in addition to glutamine synthesis.

Material and methods. A chemically defined medium known to support excellent growth of the Toronto strain and the production of high titer toxin was employed. The composition and preparation of the medium as well as the methods for cultivation are the same as previously described by Drew and Mueller(1). Total bacterial growth was estimated on the washed cells according to the Pregl micro-Kjeldahl method as adapted by Mueller(4) and expressed as "mg of bacterial nitrogen".

Experimental. Several experiments were carried out in which the growth curves produced by glutamic acid (sodium glutamate) were compared with and without ammonium sulfate added to the basal medium, and glutamine was tested as a substitute for sodium glutamate and ammonium salt. It was found that the basal medium to which sodium glutamate was added did not support good growth of the test strain and that sodium glutamate metabolism was accompanied by an accumulation of alkali (decarboxylation with the formation of (Na_2CO_3 and $NaHCO_3$)). Optimum growth took place on medium containing sodium glutamate plus ammonium sulfate, and it was observed that the presence of the

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3. McIlwain, H., *J. General Microbiol.*, 1948, v2, 186.

4. Mueller, J. H., *J. Bact.*, 1935, v29, 383.

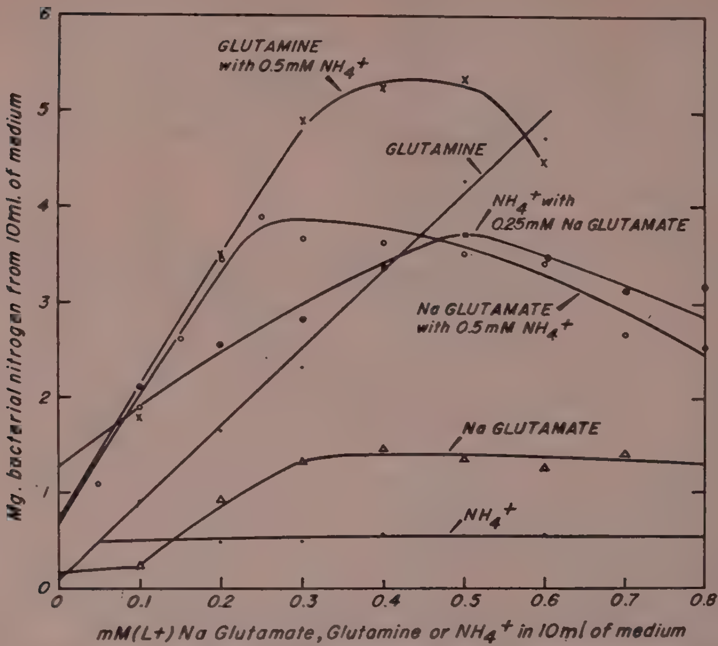

FIG. 1. Growth curves resulting from the utilization of sodium glutamate, glutamine and ammonium ion.

ammonium salt prevented the accumulation of alkali resulting from sodium glutamate metabolism. If one postulates the utilization of ammonium nitrogen and the simultaneous production of acid (H_2SO_4) as a result of ammonium sulfate metabolism, the observed neutralizing effect of the ammonium salt is readily understood. This postulation is further supported by the finding that ammonium sulfate metabolism results in the production of acid. Basal medium, to which only ammonium sulfate had been added, did not support good growth of the test organism. On this medium a small amount of growth occurred during the first 24 to 48 hours of incubation. However, cessation of growth was observed following this initial period and was attributed to the acid environment (pH 5.5-6.0). By careful selection of the amount of ammonium sulfate and sodium glutamate used, the environmental pH can be readily maintained within the range (pH 7.0-7.4) best suited to the growth of the organism. Maximum growth was obtained on basal medium containing 0.25 mM of sodium glutamate and 0.5 mM of NH_4^+.

Glutamine could be substituted in the medium for ammonium salt and sodium glutamate. Growth increased in proportion to the concentration of glutamine, and at high levels (0.5 and 0.6 mM per 10 ml medium) glutamine was more efficient than ammonium sulfate and sodium glutamate. Glutamine metabolism was not accompanied by any real accumulation of acid or alkali; the pH of the medium remained within the range of neutrality during the entire growth period. Glutamine plus ammonium ion had the greatest growth promoting effect. Fig. 1 shows the growth response to the various metabolites tested.

The ammonium salt of glutamic acid was then tested for its ability to support growth and toxin production. Ammonium glutamate was prepared by titrating a solution of l-glutamic acid with ammonium hydroxide to pH 7.0-7.2. The amount of ammonium hydroxide used was calculated to be sufficient to neutralize one carboxyl group of the glutamic acid. Growth and toxin production were determined experimentally on basal medium with added ammonium glutamate, and it was

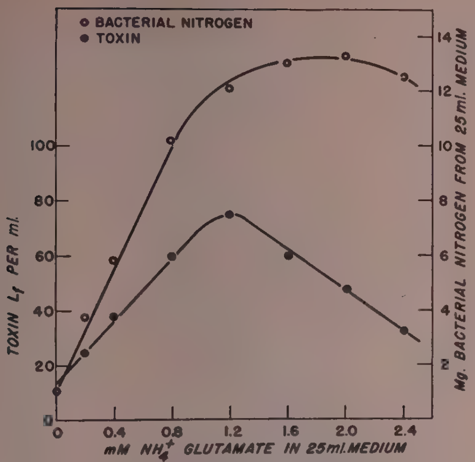

FIG. 2. Growth and toxin production on basal medium with added ammonium glutamate.

observed that the test organism grew exceptionally well on media containing 0.8 to 2.4 mM of ammonium glutamate, as evidenced by the rapidity of pellicle formation. Fig. 2 indicates that ammonium glutamate was readily utilized by the Toronto strain for growth and toxin production. Toxin production reached a maximum on medium containing 1.2 mM, but fell off sharply with increasing amounts of ammonium glutamate; the decrease in toxin production was probably due to inhibitory amounts of iron since the particular lot of glutamic acid used contained iron as impurity. The extreme sensitivity of the toxin-producing mechanism of the diphtheria bacillus to traces of iron is well recognized. In repeated experiments on iron-controlled medium, ammonium glutamate has been found to be equally as good as the combination of ammonium sulfate and sodium glutamate for growth and toxin production. Glutamine has consistently proved to be less efficient for toxin production on a low iron medium.

Discussion. The experimental results do not warrant the conclusion that glutamine is the metabolite required, and that in the absence of glutamine, ammonia and glutamic acid are essential for the synthesis of glutamine. The fact that high concentrations of glutamine are required for maximum growth makes it seem more probable that glutamine is broken down by the organism to ammonia and glutamic acid, which, of themselves, are readily utilized in a favorable pH environment. This conclusion is supported by the evidence that ammonium glutamate proved to be an efficient substitute for ammonium sulfate and sodium glutamate, or glutamine. The apparent synergism between ammonium salt and sodium glutamate may be simply a function of pH since the addition of ammonium sulfate prevents the accumulation of alkali resulting from sodium glutamate metabolism. Likewise, the acidity resulting from ammonium sulfate metabolism can be prevented by the addition of sodium glutamate.

Summary. The utilization of inorganic nitrogen in addition to organic nitrogen by the Toronto Strain of Park-Williams no. 8 *Corynebacterium diphtheriae* has been investigated. Ammonium nitrogen in the form of a simple ammonium salt (ammonium sulfate) is shown to be utilized by the organism. For optimum growth and toxin production on chemically defined medium, suitable concentrations of sodium glutamate and ammonium sulfate, or ammonium glutamate are required.

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Effect of Sanguinin, an Antibacterial Agent from Blood,* on Streptococcal Infection in Mice. (19144)

DON W. MICKS, DOROTHY M. WHITNEY, AND LUDWIK ANIGSTEIN.
From the Department of Preventive Medicine and Public Health, University of Texas Medical Branch, Galveston.

In our earlier studies(1) the antibacterial activities of tissue extracts of several species of ticks were demonstrated. It was found that the intestinal tract of blood-engorged ticks exhibited a significantly higher antibacterial titer than of those which had not been fed. Further investigations to determine the role of the blood *per se*(2) revealed that enzymatic hydrolysates of the erythrocytes of various animals showed marked antibacterial effect *in vitro*, covering a relatively wide spectrum of most gram-positive and a few gram-negative organisms. The active principle of the hydrolysate was thought to be a peptide-amino acid complex. Since this product, which has been given the name Sanguinin, strongly inhibits the growth of various organisms *in vitro* including streptococci, it was obviously of interest to ascertain its effectiveness *in vivo*.

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* Patent pend.

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This inquiry was stimulated by the observations of other investigators on the antibacterial properties of extracts from normal animal tissues(3,4) and by the growing interest in antimicrobial substances of animal origin(5). Recently, Cline *et al.*(6) studying anticatalase-producing substances, found that Sanguinin injected into mice significantly lowers the liver catalase.

Materials and methods. The following technic was used for the preparation of Sanguinin. Blood from cattle or from human blood bank was mechanically defibrinated, centrifuged, and the serum discarded. The packed cells were then submitted to dialysis according to the Anson and Mirsky(7) procedure for the preparation of hemoglobin. After freeze-drying, 30 g of the dark-red powder were suspended in 2,000 ml sodium phosphate buffer (pH 8.0) and 3 g trypsin (Pfanstiehl, 1-110) added. The mixture was then incubated for 10 hours at 37°C and heated in a 65°C water bath for 30 minutes. The material was then filtered through filter paper and the filtrate dialysed in cellulose tubing against running tap water for 48 hours and against cold distilled water for the next 24-48 hours to a pH 7.2. Sufficient N/6 HCl was added to the pooled tubing content to bring the pH to 5.0 with resulting voluminous precipitate. This is then centrifuged and the pale yellow supernate discarded. The sediment is redissolved in an adequate amount of phosphate buffer to pH 7.2 and lyophilized. This dark-brown amorphous substance is Sanguinin in its crude form. For the treatment of mice Sanguinin solution of 20 mg/ml in saline was prepared fresh daily and heated at 56°C for 20 minutes prior to administration. Subcutaneous injections were routinely given. Treatment with Sanguinin usually began one to four days before the infection and was followed after infection by daily inoculations (0.25 to 1.0 mg per mouse) for 2 or 4 days. The beta-hemolytic strain of *Streptococcus zooepidemicus* used in this study was furnished by Dr. C. E. Lankford, Department of Bacteriology, University of Texas, Austin. The cultures were maintained in Difco brain heart broth with weekly passages through mice. Broth dilutions of 1:100 to 1:1000 were prepared with the incorporation of 4% gastric mucin (Nutritional Biochemical Corp.) in early experiments until an LD50 at 3 to 4 days could be established with streptococci alone. Mice were infected intraabdominally, usually with an inoculum of 0.5 ml of a 1:1000 dilution, which dose represented an average of 300,000 bacteria. Depending upon the pretest virulence, the dosage per mouse was sometimes lowered to 0.2 ml. A total of 489 albino mice, weighing 17 to 25 g (averaging 20 g) obtained from an inbred stock were used. Serial experiments varying as to time-dose relationship of Sanguinin administration were performed on groups of 10-15 mice and accompanied by adequate controls. The latter were simultaneously and uniformly infected with the test animals.

Results. The rates of survival time of all groups of treated and untreated mice are expressed in percentages in Table I. The observations on the rapid progress of the acute

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7. Anson, M. L. and Mirsky, A. E., *J. Gen. Physiol.*, 1932, v16, 59.

TABLE I. Percentage Survival of Mice Infected with Beta-Hemolytic *Streptococcus zooepidemicus* and Treated with Sanguinin Subcutaneously.

Series	No. of mice		Sanguinin, mg/mouse			% survival on days after infection					
			Prior to infection		After infection, daily	Treated mice			Untreated controls		
	Test	Controls	Days	Daily		2nd	4th	6th	2nd	4th	6th
1	43	40	0	0	.25	88.3	23.2	6.9	89.8	22.4	2
2	30	30	1	1	.25	93.3	66.6	36.6	85.3	44.1	5.8
3	60	50	2	1	1	100	70	23	85	25.7	5.6
4	88	71	3	.75	.25	70.4	48.8	43	47.7	18.1	0
5	47	30	4	.75	.25	95.5	63.7	53.2	75	33.3	16.6

streptococcal septicemia were recorded daily and terminated on the 6th day. No treatment prior to infection was given to mice of series I, but shortly after the infection each mouse was inoculated with 0.25 mg of Sanguinin; this was continued daily over 4 days. The test mice of this series began dying approximately at the rate of the controls until the 6th day when the survival rates of the test groups were only slightly higher than in the untreated. In the next series (2, 3, 4, and 5, Table I) Sanguinin was given prior to infection. A single dose of 1.0 mg was inoculated into each mouse of the second series 24 hours before the infection. This was followed by daily inoculations of 0.25 mg beginning with the first day of infection and continuing for the next 3 days. As seen from the table, series 2, 3 and 4 were all pretreated with 0.75 or 1.0 mg/mouse daily for 2 to 4 days preceding the infection. This was followed by doses of 0.25 to 1.0 mg daily until half of the infected controls were dead. Further analysis of series 2 shows that whereas only 5.8% of the untreated controls survived the infection, 36.6% of the treated mice were alive on the 6th day. When pretreatment was begun 2 days prior to infection, the survival rates on the 6th day were 23% as compared with 5.6% of the controls. Still more significant were the results when Sanguinin was administered over 3 days before infection. The survival rate of this series rose to 43% against no survivals in the controls (series 4, Table I). Furthermore, in a 4-day pretreatment schedule of series 5, more than half of the experimental animals survived as contrasted to 16.6% in the untreated mice. The appraisal of the results expressed in survival percentages on the 6th day indicates 38.9 for the combined series of the pretreated mice against 6.0 of the total of untreated controls.

Our previous observations on the relatively high tolerance of mice to Sanguinin(2) were corroborated in the course of the present experiments. Except for an induration at the site of the inoculum and a brown discoloration of the subcutaneous tissue, no visible untoward clinical effects were observed. This refers to the dosage of 1.0 mg daily over a period of 5 days. No acute toxicity was recorded when Sanguinin was inoculated at a single dose of 5.0 mg to a mouse of 20 g body weight (J. K. Cline, unpublished data)(8).

Discussion. Its relatively low toxicity distinguishes Sanguinin from the highly toxic hematin with which it was recently identified by van Heyningen(9) and who previously found that the growth of certain aerobic sporing bacilli is inhibited by hematin *in vitro* (10). Prior to this, Kammerer(11) who was probably the first to investigate the effect of the blood derivatives on bacteria, found hematin incorporated in nutrient media stimulating rather than inhibiting the growth of various organisms. Although the presence of hematin among other components of blood hydrolysis from which Sanguinin derives is feasible, chemical evidence is lacking to identify Sanguinin with hematin.

The prolongation of survival time in all series of mice when treatment with Sanguinin began one or more days before infection, points to bacteriostatic action leading to sup-

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8. Cline, J. K., (unpublished data, 1951).

9. van Heyningen, W. E., *J.A.M.A.*, 1951, v145, 508.

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11. Kämmerer, H., *Verh. Deut. Kongr. innere Med.*, 1914, v31, 704.

pression of the disease process. Although curative treatment with Sanguinin gave only insignificant results, the survival percentages increased approximately 6-fold over the controls when the infection was preceded even by one single dose of Sanguinin (series 2, Table I). It seems, therefore, that the pretreatment is the decisive factor influencing the course of the streptococcal infection. Whereas, it is improbable that the suppression of the infection is due to a direct action of Sanguinin on the agent, it appears that the mode of action is indirectly eliciting the body defense mechanism by a non-specific stimulation. The newly established property of Sanguinin as a powerful enzymatic inhibitor(6) deserves attention in the further analysis and interpretation of its antibacterial activities.

Summary and conclusions. The blood hydrolysate Sanguinin was tested *in vivo* in 268 albino mice accompanied by 221 untreated controls for its effectiveness against the beta-hemolytic *Streptococcus zooepidemicus.* Sanguinin solution (20 mg/ml) was inoculated subcutaneously (0.25 to 1.0 mg/mouse daily, usually for 4-6 days) either prior or after infection. Suppressive effect on the course of the infection was noted in all series of treated mice. A significant increase in survival time was noted in the pretreated series with an average survival percentage of 38.9 compared with 6.0 of the total of untreated controls. It does not appear that a direct correlation can be established between the survival rates and the intake of Sanguinin or the length of its administration, however, pretreatment seems to be of decisive value.

The authors are greatly indebted to Dr. J. G. Bieri, Department of Biochemistry and Nutrition, for his valuable advice during the course of this study.

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Pharmacological Properties of a New Parasympathetic Blocking Agent, N,N Dimethyl 4-Piperidylidene 1,1 Diphenylmethane Methyl Sulfate (Prantal) (19145)

S. MARGOLIN, M. DOYLE, J. GIBLIN, A. MAKOVSKY, M. T. SPOERLEIN, I. STEPHENS, H. BERCHTOLD, G. BELLOFF, AND R. TISLOW.*

From the Biological Research Laboratories, Schering Corp., Bloomfield, N. J.

The investigations of Acheson and Moe(1), Longino, Grimson *et al.*(2,3) and Hambourger, *et al.*(4) have stimulated the search for new substances capable of blocking synaptic transmission. Interest has been furthered by encouraging clinical results reported for parasympathetic blocking agents in the management of peptic ulcer(5,6). During the study in this laboratory of a series of new quaternary salts, several compounds were found to have autonomic ganglion blocking properties. One of these, N,N dimethyl 4-piperidylidene 1,1 diphenylmethane methyl sulfate (Prantal) (7), exhibits some unusual pharmacodynamic properties which distinguish it from previously known orally active atropine-like drugs. Pharmacological data and preliminary clini-

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* The authors are indebted to S. W. Lee for valuable suggestions, and to S. Neuwirth for the biometric evaluation of data.

1. Acheson, G. H., and Moe, G. K., *J. Pharm. and Exp. Therap.*, 1946, v87, 220.

2. Longino, F. H., Grimson, K. S., Chittum, J. R., and Metcalf, B. H., *Gastroenterology*, 1950, v14, 301.

3. Longino, F. H., Chittum, J. R., and Grimson, K. S., PROC. SOC. EXP. BIOL. AND MED., 1950, v70, 467.

4. Hambourger, W. E., Cook, D. L., Winbury, M. M., and Freese, H. B., *J. Pharm. and Exp. Therap.*, 1950, v99, 245.

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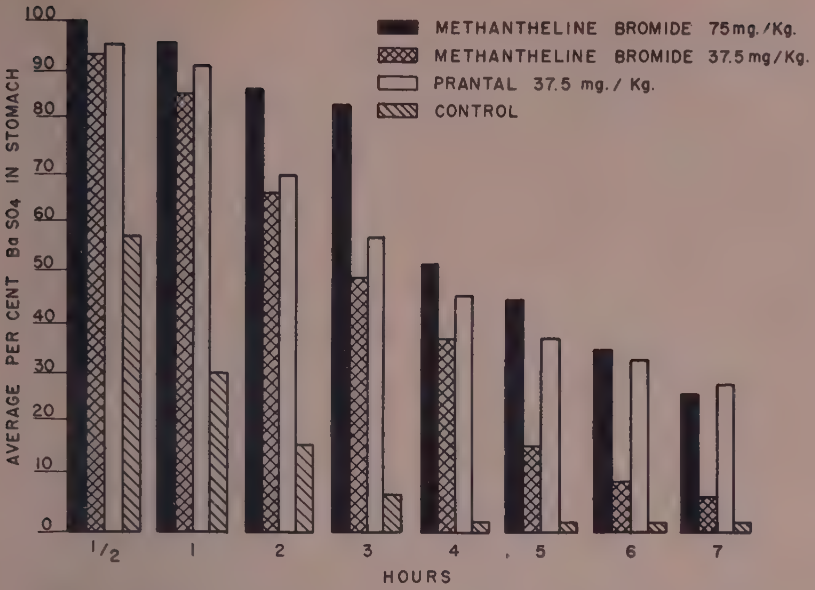

FIG. 1. Gastric emptying time following oral administration of Prantal to 4 dogs as determined by roentgenological examination after a barium sulfate meal.

cal evidence suggest that Prantal primarily acts upon the portion of the parasympathetic nervous system associated with gastric secretion and motility. In doses which elicit this selective parasympathetic blocking action, it produces no mydriasis in animals, and rarely, mydriasis or xerostomia in man.

Parasympathetic blocking action in dogs. Parasympathetic blocking effect was measured in dogs by determining the amount of Prantal required to block cardiac arrest induced by faradic stimulation of the divided vagus nerve. The cardiac arrest was completely inhibited by 0.05 mg/kg of Prantal or 0.05 mg/kg of methantheline bromide given intravenously. The methantheline bromide value is in agreement with that reported by Longino, Grimson, *et al.*(2). Both substances act for 10 to 15 minutes with this low dose, and with increasing doses the action is prolonged. *Sympathetic blocking action.* The sympathetic ganglion blocking action of Prantal was evidenced by (1) inhibition of the carotid sinus pressor reflex (increase in blood pressure during occlusion of both common carotid arteries in the dog, and (2) the block of faradic stimulation of the isolated superior cervical ganglion in the cat. The compound was injected intravenously into the femoral vein. Prantal partially or completely blocked the carotid sinus reflex in dogs when 2 to 5 mg/kg of the drug were given. Partial or complete blockade of the superior cervical ganglion in the cat also occurred with 2 to 5 mg/kg. The intravenous dose capable of blocking the sympathetic nervous system was 50 to 100 times the dose required to produce parasympathetic blockade.

Gastric emptying time in dogs. Experiments were performed with 4 trained, non-anesthetized dogs employing a technic similar to that described by Longino, Grimson, *et al.*(2). Prantal was fed in capsules at a dose of 37.5 mg/kg 75 minutes before 30 g of barium sulfate in 100 ml of water were administered by stomach tube. With the intragastric feeding of barium sulfate at "zero" time, roentgenograms were taken at ½, 1, 2, 3, 4, 5, 6, and 24 hours. Prantal was highly effective in delaying the movement of barium sulfate from the stomach; approximately 60% of the barium sulfate was still present in the stomach after 3 hours; approximately 40% after 5 hours. In control trials (no medication), barium sulfate rapidly entered the duodenum; after one-half hour approximately 60% of the barium sulfate remained in the stomach, and only 5% after 3 hours. The average of the estimates of the residual barium sulfate present in the stomach at various time intervals, as shown in Fig. 1, suggest that Prantal in these tests is more effective and longer acting than methantheline bromide.

Inhibition of gastric secretion in dogs. The effect of Prantal and methantheline bromide was studied on the gastric secretion of dogs anesthetized with chloral hydrate. Each compound was tested in 4 dogs. The gastric fluid was collected by means of a cannula passed through a duodenal incision and anchored into the pyloric orifice. Copious gastric secretion was induced by the continuous parenteral infusion of histamine dihydrochloride at the rate of 0.12 mg/kg/hour. The antisecretory action was evaluated by comparing gastric juice flow collected before and after the injection of drug. Collection of secretion was continued until the drug inhibitory effect disappeared. After the intravenous injection of 1.0 mg/kg of Prantal, the per cent reductions in gastric secretory volume for the individual dogs were 50.0%, 90.0%, 72.0%, and 53.6% (mean of 66.4%); the reductions in the total acid were 66.7%, 88.2%, 97.7%, and 44.1% (mean of 74.2%). In the dogs given 1.0 mg/kg of methantheline bromide intravenously, the per cent reductions in gastric juice flow were 88.9%, 54.5%, 71.9%, and 88.1% (mean of 75.9%); reductions in total acid were 98.0%, 60.0%, 58.3%, 86.7% (mean of 75.8%). Prantal and methantheline bromide did not differ in activity in these experiments.

Mydriatic activity. Solutions containing 0.1%, 1%, and 2% of Prantal were applied to the conjunctival sac of rabbits for a period of one minute (6 or more animals per dilution). Pupillary dilatation and impaired sensitivity to changes in light intensity occurred with the 2.0% dilution. Methantheline bromide was applied to the rabbit eye at 0.1% and 0.5% concentrations and both solutions produced mydriasis; the 0.5% dilution caused mydriasis lasting for 2 to 3 hours. The concentrations which produced mydriasis are similar to those reported by Hambourger, *et al.*(4) for methantheline bromide. In other experiments, the compounds were compared in rabbits by intravenous injection, and 1.0 mg/kg of methantheline bromide caused mydriasis in 2/20; at 2.0 mg/kg in 6/6. No mydriasis followed the intravenous administration of 2.0, 4.0, or 8.0 mg/kg of Prantal. Further evidence of the absence of mydriasis after Prantal was obtained in dog experiments described above (Fig. 1) at doses which inhibit gastric motility for several hours. In these tests, methantheline bromide induced mydriasis which persisted for 1 to 2 hours.

Specificity. The specificity of Prantal in its parasympatholytic action is indicated by several observations. The low mydriatic properties in rabbits and in dogs were mentioned above. During the x-ray studies with dogs, it was observed that Prantal exhibited little delaying action upon the transit time of a barium sulfate meal through the small intestine although stomach emptying time was greatly delayed. In anti-acetylcholine experiments *in vitro* (modified Magnus-Dale technic) the median effective concentration for Prantal was 10.75 μg/liter, and 1.25 μg/liter for atropine sulfate. These dilutions caused a 50% inhibition of maximal spasm of guinea pig ileum induced by 20.0 μg/liter of acetylcholine chloride. The intravenous injection of 0.05 mg/kg of methantheline bromide inhibited the vasodepressor response in dogs to intravenously administered acetylcholine chloride (0.1 mg/kg); whereas, 0.4 mg/kg of Prantal was required to obtain the same degree of inhibition. In the previously cited experiments demonstrating the ability of Prantal (0.05 mg/kg) to block cardiac arrest induced by faradic stimulation of the divided vagus nerve, Prantal and methantheline bromide did not differ in activity. *In vivo,* orally administered Prantal prevented bronchospasm and death in guinea pigs caused by the intravenous injection of an otherwise lethal dose of acetylcholine chloride (14 mg/kg). The median effective dose for Prantal was 27 mg/kg; for oral methantheline bromide, 62 mg/kg. The tremors, convulsions, and death induced in mice by subcutaneous injection of 2.0 mg/kg

TABLE I. Oral Acute Toxicity of Prantal in Rodents.

Species	No. of animals	LD_{50}, mg/kg	Confidence limits (P=.05), mg/kg
Mouse (Manor)	64	317	269 to 361
Rat (Rockland)	30	1107	900 to 1362
Guinea pig (Manor)	18	404	254 to 642

of physostigmine were antagonized by subcutaneous atropine sulfate (ED50 5.0 mg/kg); the subcutaneous ED50 for Prantal was 85 mg/kg, and for methantheline bromide, 13 mg/kg. Antihistaminic experiments *in vitro,* and tests *in vivo* with guinea pigs did not demonstrate any significant antihistaminic activity for Prantal.

Acute toxicity in rodents. The oral acute toxicity for Prantal was determined in the mouse, rat, and guinea pig, and values are listed in Table I. Graded doses of aqueous preparations were fed by stomach tube or oral syringe to groups of 6 or more animals. Mortality, over a 5-day period, was recorded, and the median lethal dose (LD50) with confidence limits was computed by the method of Litchfield and Wilcoxon(8). The intraperitoneal median lethal dose (LD50) in 18 to 22 g non-fasted mice was 47 mg/kg. In mice, rats or guinea pigs death was usually caused by respiratory depression and collapse.

Acute intravenous toxicity in dogs (infusion). Mongrel dogs were anesthetized with chloral hydrate or sodium pentobarbital, and the carotid blood pressure, pulse and respiration rate were recorded. Prantal was infused at an average rate of 0.280 mg/kg/min into the right femoral vein of 5 dogs weighing 4.1 to 6.0 kg; a mean lethal dose of 41.6 mg/kg was found. No significant change in the respiration rate or blood pressure was seen until just prior to death; pulse rate decreased steadily until death. Death was due to respiratory failure. Intravenous infusion of methantheline bromide into 4 dogs weighing 8.0 to 9.0 kg at an average rate of 0.300 mg/kg/min caused a distinct drop in blood pressure with the onset of the infusion, and this continued until death. A drop in the pulse rate was seen; no significant change in the respiration rate occurred until just prior to death. The mean lethal dose for methantheline bromide was 22.7 mg/kg. These data indicate Prantal to be approximately ½ as toxic as methantheline bromide by intravenous infusion in the dog.

Safety. Acute toxicity data for several species indicate a high safety ratio between toxic and parasympatholytic dosages. In acute intravenous studies with dogs, Prantal was less vasodepressor than atropine sulfate, or methantheline bromide. Subacute toxicity tests in mice, chronic experiments in rats, and detailed chronic studies with large over-dosages in dogs have not shown cumulative toxicity. During these investigations, growth rate and hematological findings were normal. At autopsy, no gross pathological changes were seen, and histopathological study of various organs disclosed no toxic effects.

Antidote. Experiments conducted in dogs with intramuscular or intravenous Prantal overdosage showed that the first sign of intoxication was tachycardia, followed by mydriasis, central nervous system depression, and respiratory depression. In tests with dogs, physostigmine intramuscularly (1 mg/kg) was found to be a satisfactory antidote when used even after the onset of respiratory depression. Supportive artificial respiration was also applied when warranted.

The parasympatholytic activity and low toxicity in experimental animals appeared sufficiently encouraging to warrant evaluation of Prantal in man. Preliminary results of oral or parenteral administration of test doses in 24 patients with peptic ulcer and of continuing treatment in 17 indicate therapeutic usefulness and minimal side effects (Grimson, Flowe, Rowe, and Emlet) (9). Gastric motility is inhibited an hour and longer following 50 mg intramuscularly or 100 mg orally. Gastric secretions are usually reduced in volume with marked lowering of total and free hydrochloric acid titers following 50 mg intramuscularly. Reduction is variable after a

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8. Litchfield, J. T., and Wilcoxon, F., *J. Pharm. and Exp. Therap.*, 1949, v95, 99.

9. Grimson, K. S., Flowe, B. H., Rowe, C. R., and Emlet, J. R., Personal communication.

single oral dose. Continuous oral treatment using 100 mg(9) or 50 to 100 mg(10) every 4 or 6 hours usually relieves symptoms of ulcer.

Summary. (1) A new chemical compound has been found which has high oral and parenteral parasympathetic blocking activity. This compound, N,N dimethyl 4-piperidylidene 1,1 diphenylmethane methyl sulfate (Prantal), is unique in its specificity of action within the parasympathetic system, *i.e.*, it primarily inhibits gastric motility and gastric secretion. The intravenous sympathetic ganglion blocking dose is 50 to 100 times that required for intravenous parasympathetic blockade. (2) In dogs, roentgenologic observations of the movement of barium sulfate from the stomach indicate that orally administered Prantal delays gastric emptying time more effectively, and is longer acting than methantheline bromide. Additional dog experiments showed the ability of Prantal to reduce the volume and titratable total acid of gastric secretions. (3) After oral medication in dogs, Prantal produced no mydriasis at doses which inhibit gastric motility for several hours, whereas methantheline bromide caused mydriasis which persisted for one to 3 hours. In rabbit tests by topical application to the eye, mydriasis occurred with a 0.1% concentration of methantheline bromide, whereas 1.0% of Prantal was not mydriatic; mydriasis occurred at the 2.0% concentration of Prantal. By intravenous injection in rabbits methantheline bromide caused mydriasis at 1.0 and 2.0 mg/kg doses; no mydriasis occurred at 2.0, 4.0 or 8.0 mg/kg doses of Prantal.

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Regulation of Breathing in Man at Altitude.* (19146)

F. G. HALL. (With the technical assistance of Charles Foreman.)
From the Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, N. C.

Peracute hypoxia occurs in an aviator at high altitude if he loses his oxygen supply or if he suffers sudden decompression. The time that he will retain consciousness depends upon several factors, some of which have been reported(1). It has been shown that the addition of carbon dioxide to the inspired air increases the consciousness time of man at altitude(2). This investigation has been extended for the purpose of studying the relative influence of hypoxic and carbon dioxide stimuli upon pulmonary ventilation in the peracute hypoxic state. A simulated altitude of 25,000 feet was selected for the reason that in previous tests it was found that this altitude was somewhat marginal in its effect upon consciousness, the loss of which occurs only after several minutes. This allows sufficient time to record pulmonary ventilation for a few minutes and permits any respiratory change to reach an approximate steady state. Thus the pattern of the rapid hypoxia response can be more clearly observed.

Procedure. Ten experimental subjects were used in this investigation. They were medical students, physically fit and in good health. All had previous experience in the low pressure chamber during exposures to simulated high altitude. From 4 to 6 separate tests were made on each subject. Pulmonary ventilation was determined from photokymographic records as previously described(1). Thus a continuous record of pulmonary ventilation was obtained. Standard Air Force A-13 oxygen masks were used. These were fitted to prevent leakage and a leakage test was

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* Work done under contract with the Aero Medical Laboratory, Air Research and Development Command, U.S.A.F.

1. Hall, F. G., *J. Applied Physiol.*, 1949, v1, 496.

2. Hall, F. G., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 487.

made during each experiment. No leakage greater than 1% was observed. Each subject was instructed and trained in manipulation of the useful consciousness tester as previously described(1). A continuous record of each subject's response was obtained for the full duration of each test.

Each subject was brought to a simulated altitude of 25,000 feet (282 mm Hg.) while breathing 100% oxygen. After allowing 2 minutes for adjustment to this altitude the subject was switched from oxygen to one of 3 gas mixtures. He was kept on the gas mixture for 5 minutes and then returned to 100% oxygen. Three gas mixtures were used: A, 20.9% O_2, 0.04% CO_2, 79% N_2; B, 88% O_2, 11.6% CO_2, 0.4% N_2; C, 20.9% O_2, 11.6% CO_2, 67.5% N_2. Gas analyses were made on each gas mixture during each individual test by the standard Haldane procedure. Tests were made to ascertain if the sequence of using the various gas mixtures was important. The mixture of 88% O_2 and 11.6% CO_2 was used in the first and last test. Results showed that the sequence was not important.

Results. The results of 40 simulated flights with 10 subjects are summarized in Fig. 1. In each test the pulmonary ventilation was measured for the period during which the subject was breathing 100% oxygen and then for the subsequent period in which he was breathing a particular gas mixture. The determinations were made for one minute intervals and the pulmonary ventilation ratios were calculated by defining the ventilation during breathing of 100% oxygen as unity. Because of variations in pulmonary ventilation among individuals it was considered more desirable to use ventilation ratios and have each subject his own control. The results shown are the mean values obtained for ten subjects.

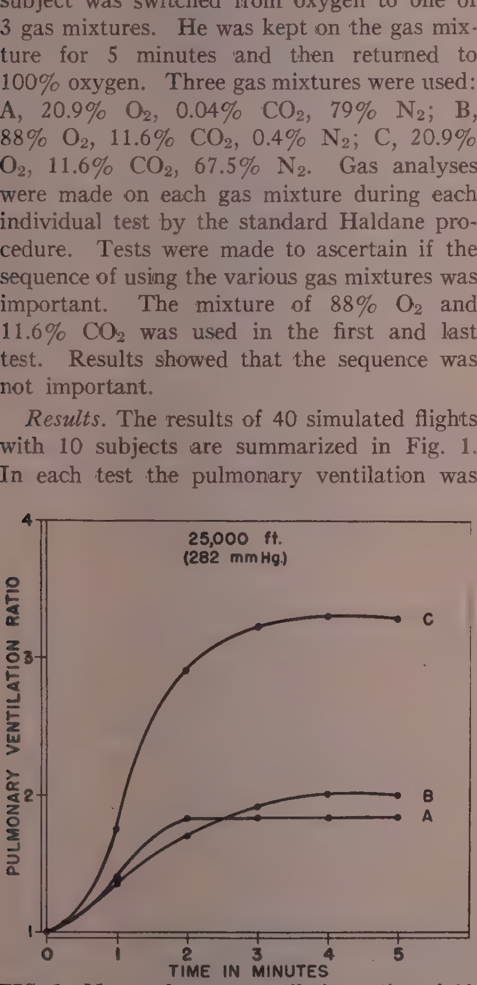

FIG. 1. Mean pulmonary ventilation ratios of 10 subjects breathing various gas mixtures at a simulated altitude of 25000 feet (282 mm Hg). The composition of inspired air in curve A, O_2 20.9%, CO_2 .04%, N_2 79%; curve B, O_2 88%, CO_2 11.6%, N_2 .4%; curve C, O_2 20.9%, CO_2 11.6%, N_2 67.5%.

It will be observed from inspection of Fig. 1, that at a simulated altitude of 25,000 feet the hypoxic "drive" of a subject breathing ambient air causes an increase in pulmonary ventilation approximately equal to that when he is breathing a relative high concentration of oxygen along with 11.6% CO_2. When, however, the oxygen concentration equal to that of the ambient air is breathed with 11.6% CO_2 a much higher pulmonary ventilation occurs.

Responses to hypoxic and carbon dioxide stimuli were fairly rapid as can be seen in curve A and B respectively. In both cases a steady level of pulmonary ventilation was maintained after 2 minutes.

It can be questioned whether or not the difference in the nature curve A and B is significant. Perhaps a plausible explanation is that due to the low oxygen reserve of the transport system the hypoxic stimulus operates quickly while the CO_2 stimulus acts more slowly because of the buffering effect of the alkaline reserve.

It should also be mentioned that in 4 of the 10 subjects breathing ambient air consciousness was lost a few seconds before the end of the 5 minute period. This did not occur when carbon dioxide was added to the inspired air. This is further confirmation of previous findings(2) at higher altitudes.

Discussion. Otis *et al.*(3) have pointed out that hypoxia and hypocapnia are additive in their effects upon performance. Gray(4) has theorized that pulmonary ventilation is determined by the algebraic sum of separate

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3. Otis, *et al. Am. J. Physiol.*, 1946, v146, 207.
4. Gray, J. S., *Science*, 1946, v103, 739.

factors. The report by Dill and Zamcheck (5) give quantitative experimental evidence of the effects of adding carbon dioxide to inspired air deficient in oxygen at sea level. These authors concluded that the addition of carbon dioxide to inspired renders a low partial pressure more tolerable by increasing arterial saturation. The results presented herewith are in agreement with these authors. They also show the rapidity of the response to hypoxia and hypercapnia, and something about the interaction of these two factors. These results would seem to indicate that the potency of the hypoxic stimulus is not fully manifested when man breathes ambient air at altitude. In Fig. 1, curve A shows the ventilatory response to breathing ambient air which involves both hypoxic and hypocapnic stimuli. Curve B, shows the ventilatory response to hypercapnia at the same altitude but with adequate oxygen for full saturation of blood. Curve C, shows the additive effect of hypoxia and hypercapnia. It would seem that the difference in the ventilatory response shown in curves B and C must be due to the hypoxic stimulus since the concentration of carbon dioxide in inspired air is the same. Of course the p CO_2 of alveolar air will be slightly lower in C due to the increase in ventilation and the p O_2 will be slightly higher. Thus these two interacting factors appear additive in their effects.

Summary. When man breathes ambient air at high altitudes an hypoxic stimulus causes a rapid increase in pulmonary ventilation. The full effect of this hypoxic stimulus is counteracted, however, by concomitant hypocapnia. With the addition of carbon dioxide to inspired air a more effective response to the hypoxic stimulus is manifested. It is concluded that hypoxia and hypercapnia are separate stimulating factors and additive in their effects and that regulation of respiration at altitude depends upon the manner of their interaction.

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5. Dill, D. B., and Zamcheck, N., *Am. J. Physiol.*, 1940, v129, 47.

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Effect of Electroconvulsive Shock on Body Temperature of the Rat. (19147)

RUTH HOYT AND H. ENGER ROSVOLD.* (Introduced by Donald McEachern.)
From the Department of Psychology, McGill University.

Rosvold(1,2) suggested that elevation in body temperature may have contributed to the disruptive effect of electroconvulsive shock on nest building behavior in rats. The present study was designed to demonstrate the effect of electrically induced convulsions on the temperature of the rat.

Subjects and method. Twenty male hooded rats 150-180 days of age were divided evenly into a control and an experimental group. They were placed 2 to a cage size 8" x 9" x 11" in which fox chow and water were available at all times, and were housed in a room in which the temperature was maintained between 70-80°F. A convulsion was induced in the experimental animals at 9 a.m. daily during the shock periods by administering a 50 milliampere current for 0.2 second through clip electrodes attached to the ears. The electrodes were fastened to the ears of the control animals, but no current was delivered. By inserting a thermometer eight and one-half centimeters into the rectum of the restrained rat for 2 minutes, temperatures were taken on 2 different animals from each group at 9 a.m., 12 n., 3 p.m., 6 p.m., and 9 p.m. The temperature of any particular animal was taken at a different time on successive days, no animal being examined at the same time in a 5-day period. The following schedule was followed:

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* Now at the Yale University School of Medicine, Department of Physiology.

1. Rosvold, H. E., *J. comp. and physiol. Psychol.*, 1949, v42, 118.

2. Rosvold, H. E., *J. comp. and physiol. Psychol.*, 1949, v42, 207.

1. 5 days of no shock for either group to establish a base line.
2. 10 days during which the convulsed group received daily shocks.
3. 10-day recovery period. No shocks.
4. 10-day period of daily shocks.
5. 10-day recovery period. No shocks.

Results. Fig. 1 shows the mean temperature at the specified hours for the shocked and for the non-shocked animals during the shock and no-shock periods. The difference between the means of the temperatures of the shocked animals and the control animals during the shock periods at 12 noon, 3 p.m., and 6 p.m., is significant at the .01 level of probability; at 9 a.m., the .05 level; and at 9 p.m., the .75 level. The difference between the means of the temperatures of the shocked animals in the shock and no-shock periods at 3 p.m. and 6 p.m. is significant at the .01 level of probability; at other times at less than the .05 level.

The differences in the mean temperature of the shocked and non-shocked animals are reliable only at those times during the day when rats are normally inactive. Instead of dropping, the temperature of the convulsed rats remains at the high level of the active period suggesting that the electroshock results in decreased variability in temperature. That this is indeed the case is apparent from a comparison of the variances of the mean temperatures of the shocked and control animals during the shock periods. The F ratio for this comparison is 14.9, which is significant at the .02 level of probability indicating the variability of the mean temperature of the shocked rats to be significantly less than that of the non-shocked rats.

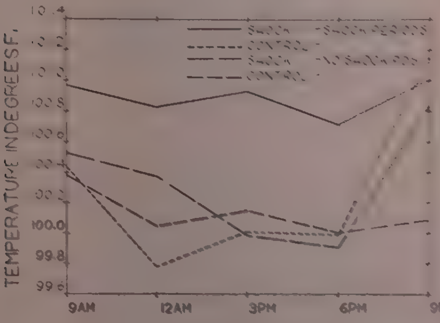

FIG. 1. Differences in mean temperature of shocked and non-shocked rats.

In order to rule out the possibility that increased activity in the shocked rats was maintaining the temperature at this high level, activity in revolving drums was measured in 5 hooded rats age 150-160 days of age. Readings were taken for 30 days at the same hours as temperatures were taken in the groups above. For 10 days no shock was administered, then for 10 days one shock was administered daily at 9 a.m., then for 10 days no shock was administered. During the shock periods there was a depression of activity significant at the .01 probability level as compared with the no-shock periods. These findings are substantiated by those found for the white rat by Winder and Stone(3). Since inactivity would tend to decrease temperatures, the elevated temperatures in the shocked rats cannot be attributed to changes in the activity cycle.

Conclusion. A series of electroconvulsive shocks results in decreased variability of the temperature of the rat. This decreased variability is probably not the result of increased activity, but is more likely attributable to a failure of a temperature regulation mechanism.

Discussion. Ranson(4) has shown temperature regulation, and Nauta(5), maintenance of activity, to be controlled in the hypothalamus. Since both these functions are disturbed by electroconvulsive shocks, it is reasonable to suppose that the electroshocks affect the hypothalamus.

Summary. The normal variation of rectal temperature in the hooded rat has been shown to be decreased during periods of electroconvulsive shocks. It is suggested that electroconvulsive shocks may affect the temperature regulation mechanism of the hypothalamus.

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Chemical Stimulation and Inhibition of Bacterial Motility Studied with a New Method.* (19148)

EDUARDO DE ROBERTIS AND CIRO A. PELUFFO.

From the Departamente de Ultraestructura Celular, Instituto de Investigacion de Ciencias Biologicas, and Departamento de Bacteriologia, Instituto de Higiene, Montevideo, Uruguay.

In a recent electron microscope study of purified flagella from *Bacillus brevis* it was shown that they are composite protein structures with a trypsin-resistant axial filament and a trypsin-sensitive peripheral sheath (De Robertis and Franchi) (1,2). The axial filament has a highly complex helical ultrastructure that seems to be particularly well adapted to the mechanism underlying the motility of bacteria flagella. By studying the action of different chemical agents on this protein it was found that adenosintriphosphate (ATP) causes the contraction of the flagellar filaments while other organic and inorganic phosphate compounds have no visible influence. Furthermore, it was observed that the thiol inhibitors—p-chloromercuribenzoate and iodosobenzoate—interfere with the action of ATP (2).

These findings suggested the study of the action of these and other chemical agents on the motility of the living bacteria. The problems involved in bacterial locomotion have been recently discussed by Weibull(3). Fleming *et al.*(4,5) observed that in bacteria grown in sub-lethal concentration of penicillin motility is apparently influenced by radiant heat. However, the source of energy and the probable enzymatic mechanisms involved in bacteria locomotion were not investigated. In the present study several chemical agents known to act on specific groups present in proteins or on particular enzymatic processes were used. Special care was taken to follow the action of different drugs added to groups of bacteria previously observed in normal locomotion. For this purpose a special chamber and culture technic was developed. This method allows for prolonged and undisturbed observation of a group of bacteria while they are submitted to the action of different agents.

Material and methods. *Proteus vulgaris*, H × 19 was grown on semisolid agar to increase the motility and then transferred to plain agar the day before the test. The culture chamber was prepared as follows: Grooved slides were made by sticking down 2 cover-slip strips on a slide leaving between them a channel, 3-5 mm wide (Fig. 1). Cellophane strips (24 × 28 mm) were sterilized by boiling and afterwards kept in a Petri dish with sterile broth. To set a chamber, a thin film of a 24-hour culture of *P. vulgaris*

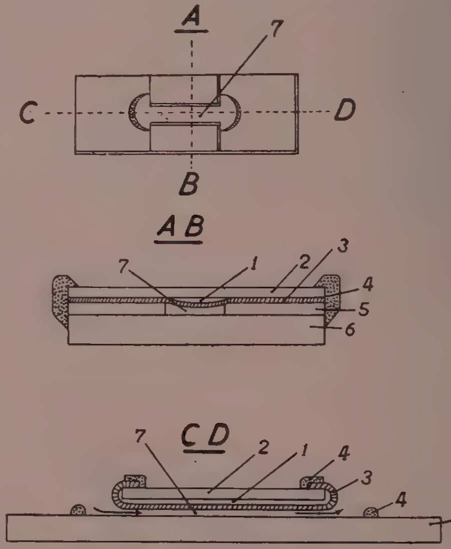

FIG. 1. Diagram of the culture chamber. *Upper*, view from above, *middle* and *lower* sections into lines A B and C D (see description in the text.) 1. culture, 2. cover-slide, 3. cellophane membrane, 4. wax, 5. cover-slide strip, 6. slide, 7. channel.

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* This work was supported by a grant of the Rockefeller Foundation.

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TABLE I. Action of Different Inhibitors on the Motility of *Proteus Vulgaris* HX 19.

Inhibitor	Molar concentration	Degree inhibition	Time starting inhibition, sec	Time of max inhibition, sec
p. chloromercuribenzoate	.001	4+	30	180
Mercury dichloride	.01	4+	30	60
Etilmercurithiolsalicilate ''merthiolate''	.025	3+	30	180
Iodosobenzoate	.002	3+	30	300
Iodoacetamide	.01	2+	30	90
Iodoacetate	.01	1+		180
Potassium cyanide	.001-.01			
Sodium azide	.01			
Malonate	.01			
Sodium fluoride	.01			
Chloroform	.08	3+	30	60
Chloral hydrate	.3	4+	20	60
,, ,,	.14	3+	40	180
,, ,,	.06	2+	40	180
Barbital	.33	3+	20	240
Phenobarbital	.1	3+	15	30
Metadon	.03	3+	30	120

1+, translation slowed down, vibration persists; 2+, a few bacteria with translation, vibration persists; 3+, translation stopped, some vibration *in situ;* 4+, total paralysis.

was spread over the middle zone of a sterile cover-slip and then kept in a Petri dish until it dried out. A cellophane strip was stretched cross-wise on top of a slide, and a loopful of melted plain agar was dropped on its center. Then the cover-slip, carrying the culture, was quickly inverted over it. After setting, the cellophane flaps were turned up on both ends and the chamber taken off from the supporting slide and laid, with the cellophane down, over the grooved slide. The chamber was pressed down with a piece of filter paper and, as soon as the sides were dry, was sealed with wax leaving both ends of the channel open. To prevent the spreading of the solution on the slide a narrow ring of wax was put around each open end of the channel. The inoculated chambers were kept moist in a Petri dish, over a layer of damp cotton. After 24 hours incubation at room temperature, the channel was filled with saline, and the chamber examined under a dry objective (N.A. 0.85) with the phase-contrast microscope. A place where the bacterial density was optimal and with no liquid currents was selected for the experiments. In some chambers it was possible to find small "swimming pools" with a few motile organisms that could be followed individually. The solution to be tested was dropped at one end of the channel and it ran easily by capillarity to the opposite end. The rate of flowing could be increased by absorbing the emerging liquid with a piece of filter paper. The drugs were generally dissolved in buffer phosphate of pH about 7 at the concentrations indicated in Tables I, II, and III. All together about 100 chambers were examined and submitted to the action of different drugs. In some cases only one substance was investigated in a single chamber but other times the action of two or more drugs were successively applied to the same group of bacteria.

Results. Action of different inhibiting drugs on the motility of Proteus vulgaris. Table I summarizes the main results obtained with different inhibitors on the motility of *Proteus vulgaris.* The degree of inhibition was estimated by the changes in motility until complete paralysis occurred. Although there were differences from one culture to another, probably related to variations in the chamber, age of the bacteria and so forth, in general the inhibition of motility followed quite a definite pattern. For example, with chloral hydrate (0.3 M) the first reaction was observed after 10 to 20 seconds when the movement of translation in the long forms of bacteria was diminished and finally stopped. A few seconds later translation of the medium size and short bacteria was also inhibited though they still showed vibration *in situ.* Finally a complete inhibition of motility was observed.

TABLE II. Reversal of Inhibition Produced by Thiol Inhibitors on the Motility of *Proteus Vulgaris* X 19.

Thiol inhibitor	Degree of inhibition	Thiol-reducing agents, M .005	Time starting to act, sec	Time max reversal	Degree of reversal
p. chloromercuribenzoate M .001	4+	Glutathione	20	60	Complete
		Cysteine	30	60	”
		Ascorbic acid			
Mercurichloride M .001	4+	Glutathione	30	180	Almost complete
Etilmercurithiosalicilate M .025	3+	”		90	Complete
Iodosobenzoate M .002	3+	Cysteine	60	150	”
Iodoacetamide M .01	2+	Glutathione			”
Iodoacetate M .01	1+	”		240	”

TABLE III. Action of ATP and Other Phosphate Compounds After the Inhibition of Motility with M .3 Chloral Hydrate for 60 Seconds.

Exp. No.	Treatment	Starting vibration, sec	Starting translation, sec	Starting vibration, sec	Starting translation, sec
11	Saline		300		150
29	Buffer pH 7	360		40	
30	Glucose 1-6 phosphate	240		120	
13	Glucose 1-6 phosphate	300		120	180
18	3 phosphoglyceric acid	120	180	80	150
18 bis	Sodium pyrophosphate	120	300	90	220
17	Buffer pH 7	120		40	
53	Na pyrophosphate	120	360		220
59	3 phosphoglyceric acid	90	540	120	300
55	Glucose 1-6 phosphate	150	270	120	210
83	Saline	210	360	40	90
92	”		600		300
97	”		510		360
Means		183	380	85	218

Action of thiol inhibitors. Table I shows that the strongest inhibiting action was observed with the mercaptide-forming agents: p-chloromercuribenzoate acting at a concentration 0.001 *M* and mercuric chloride at 0.01 *M*. Ethyl mercurithiosalicilate (Merthiolate), probably acting by a similar mechanism, produced also a considerable inhibition, although not complete, at a concentration of 0.025 *M*. The oxidizing agent iodosobenzoate had less inhibiting action, while the SH alkylating drugs, iodoacetamide and iodoacetic acid, had a very small inhibiting action. The specificity of the action on thiol groups was confirmed in all cases by reversal of the inhibition with thiol-reducing agents. As shown in Table II, the action of all SH inhibitors was rapidly reversed with 0.005 *M* glutathione or cysteine and normal motility was restored. Simple washing with saline or buffer solution did not reverse the action of the thiol inhibitors while the effect of the reducing agents was in some cases instantaneous.

Action of narcotics and other inhibitors. Agents known to act on the oxidative mechanisms of bacteria, such as potassium cyanide and sodium azide, had no visible action on the motility of the bacteria during the period of observation. Results with malonate and with sodium fluoride were also negative. Substances having a narcotic effect, such as chloral hydrate, sodium barbital and chloroform, inhibited motility at high concentrations. One of the most active seems to be chloroform, while the least active seems to be barbital. Phenobarbital was more active than barbital and its action was rapidly reversed by washing. Complete inhibition with chloral hydrate required a concentration 300 times higher than with p-chloromercuribenzoate. In addition to this difference the inhibition by these narcotics was restored by simple washing. Metadon (chlorhydrate of 4-4 diphenil 6 dimethyl aminoheptenone) had the strongest effect and, after acting for 4 minutes, its inhibition could not be reversed. The recovery depended, among other factors, on the concentration and on the time of action of the narcotic. In Table III are recorded the periods of time after washing before vibration and translation started. In these cases chloral hydrate 0.3 *M* had previously acted for 60 seconds. Complete recovery of motility took, however, much longer periods. In 2 experiments with phenobarbital the motility was apparently restored more rapidly with amphetamine sulfate (Benzedrine) 0.03 *M* than with buffer.

Action of adenosintriphosphate. In several experiments it was observed that ATP had a definite stimulating effect on the motility of *Proteus vulgaris*. This was particularly clear in some slow forms of this bacillus. However, the action was difficult to detect in rapidly moving bacteria. In order to obtain results which could be observed objectively and expressed numerically the following method was used. Bacteria were paralyzed completely by the action of chloral hydrate (0.3 *M* for 60 sec.) and a record was taken of the time in which vibration and translation started after washing with saline buffer of pH 7 (Table III). Afterwards the same bacteria were treated again with chloral hydrate and the recovery was obtained by washing them with ATP or other phosphate compounds. Since the effect of the second treatment with the narcotic is normally more prolonged than the first, ATP was always applied after the second inhibition. As indicated in Table III, in spite of this handicap ATP induced recovery of vibration and translation in a much shorter time than the buffer, the saline solution, or the phosphate compounds: Sodium pyrophosphate, 3-phosphoglyceric acid, glucose 1-6 phosphate, and adenylic acid (the latter not indicated in the table). Although the times varied considerably from one experiment to another, the differences are clear. With ATP vibration started after a period about 110% shorter than in the controls while translation started after a period 74% shorter. The activating action of ATP was inhibited by a previous treatment with a strong thiol inhibitor such as p-chloromercuribenzoate.

Discussion. The special technic described here may open a new way for obtaining information about the fundamental factors involved in bacterial motility since it makes it possible to observe for any length of time the motility of individual bacteria submitted to the action of different chemical or physical agents. The use of a chamber with permeable wall has many other possibilities since it is possible in this way to study the effect of chemical agents on actively multiplying cells as well as on "resting suspensions." Problems of bacterial growth and multiplication could also be investigated. The use of cellophane membranes in the culture of microorganisms has been already described, but only with the purpose of obtaining high concentrations of bacteria in a small volume(6). This membrane is permeable to drugs of small molecular weight, like those used in this work. Although the rate of diffusion across the membrane was not investigated, the results obtained with narcotics or with —SH reagents (the latter showed activity in about a few seconds) point to a fairly fast action. Negative results with drugs of a similar molecular weight could not possibly be due to lack of diffusion.

The reversal of the inhibitory action of several compounds by simple washing with saline or by the use of specific reducing agents indicates that it is not safe to take inhibition of motility as a direct manifestation of lethal effect. Buchbinder and Zaretsky's test(7) for the determination of the bactericidal concentration of quaternary ammonium compounds cannot therefore be accepted without further

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analysis as a general test for bactericidal action.

Motility of bacteria and SH groups. Experiments on the action of thiol inhibitors indicate that —SH groups are probably involved in the motility of *Proteus vulgaris.* Among those inhibitors the mercaptide-forming agents had the strongest action and could stop the bacterial movement entirely. Of these agents p-chloromercuribenzoic acid has the advantage of combining with single R-SH groups and of being, according to Barron(8), "the most powerful inhibiting agent and the most specific when accompanied by reactivation." The other thiol inhibitors of the oxidizing type and particularly those having an alkylating action such as iodoacetate and iodoacetamide had a smaller effect. Similar differences are often found in —SH systems (8). The action of these inhibitors could not be reversed by simple washing in buffer or saline, while motility was immediately restored with the specific reducing agents of the SH groups: glutathione and cysteine. The action of thiol inhibitors on bacterial motility may probably be interpreted as evidence for the presence of essential —SH groups in the contractile protein of bacterial flagella or in enzyme specially involved in the mechanism of flagellar contraction. In favor of this hypothesis are also the electron microscope observations of De Robertis and Franchi(2) showing that SH inhibitors interfere with the contracting action of ATP on the purified flagellar protein of *Bacillus brevis.* This action is also similar to that previously observed in contractile proteins obtained from muscle. Barron and Singer(9) found that p-chloromercuribenzoate completely inactivated the ATPase activity of myosin while alkylating agents such as iodoacetate and iodoacetamide had no action. Furthermore, Bailey and Perry(10) demonstrated that SH groups are essential for the formation of actomyosin. In view of these facts it would be of interest to study the ATPase activity of bacteria and to see if it is present in purified flagella.

Motility of bacteria and ATP. Nothing is known about the source of energy used by flagella during contraction. The lack of effect on motility by compounds known to have an inhibitory action on the oxidative cycle or on glycolysis might be attributed to the metabolic characteristics of *Proteus vulgaris.* It seems probable that a different effect may be found in bacteria having other mechanisms for obtaining energy. In our experiments the only agent found to have a definite stimulating effect on the motility of *Proteus vulgaris* is adenosintriphosphate. All the other inorganic and organic phosphates employed had no visible action on locomotion. Furthermore, our previous electron microscope observations (2) show that flagellar protein contracts under the action of ATP, but not with other phosphate compounds. Since the discovery of the ATPase activity of myosin(11) and the demonstration that ATP induces contraction on actomyosin(12) the role of this compound on muscle contractility has been extensively investigated. ATP seems to have also an important role in the motility of sperm (Lardy *et col.*)(13). The results reported here show that ATP may be also one of the sources of energy involved in bacterial motility and in the contraction of bacterial flagella.

Conclusions. (1) The motility of *Proteus vulgaris* × 19 has been studied in a special chamber which permits prolonged and undisturbed observation of a group of bacteria under the influence of different chemical agents. (2) Strongest inhibition was observed with thiol inhibitors of the mercaptide-forming type, particularly with p-chloromercuribenzoate. The oxidizing agent iodosobenzoate and particularly the alkylating-SH drugs, iodoacetamide and iodoacetate, had a

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13. Lardy, H. P., Hansen, R. G., and Phillips, P. H., *Arch. Biochem.,* 1945, v6, 41.

less marked inhibitory action. The specificity of the action of thiol inhibitors was confirmed in all cases by reversal of the inhibition with the thiol-reducing agents: glutathione and cysteine. Potassium cyanide, sodium azide, sodium fluoride and malonate had no action on the motility of *Proteus vulgaris*. (3) Substances having a narcotic effect, such as chloral hydrate, barbital, phenobarbital and chloroform, inhibited motility only at high concentrations. In these cases recovery of motility could be obtained by simply washing in buffer or saline. (4) Adenosintriphosphate had a definite stimulating effect on the motility of *Proteus vulgaris*. It was found that in bacteria inhibited with chloral hydrate vibration and translatory movements started within periods of time which were 110 and 74% shorter with ATP than with saline or other organic or inorganic phosphates. The possible role of —SH groups and of ATP in bacterial locomotion is discussed.

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Size and Shape of Blood Group *A*-Substance. (19149)

Arthur B. Pardee* and Robert H. Blaker.† (Introduced by W. M. Stanley.)

From Gates and Crellin Laboratories of Chemistry, California Institute of Technology.

Kekwick has recently presented data on the size and shape of an A-substance preparation from pseudomucinous ovarian cyst fluid(1). He obtained a molecular weight of 260000 and an axial ratio of 60/1 from ultracentrifuge and diffusion measurements. For comparison it seems worthwhile to present briefly data obtained on other preparations of A-substance(2).

Materials. The closely related preparations R18-F10, R19-F1A, and R19-F2A of A-substance from hog gastric mucin were used (3,4).‡ This material is known to be chemically and immunologically inhomogeneous (3,4). The solvent used in all experiments was 0.02 M phosphate buffer at pH 7.1 with sufficient sodium chloride to give an ionic strength of 0.15.

Light scattering. Measurements were made at concentrations between 1.0 and 0.1%. Values of the reciprocal of specific turbidity were plotted against concentration and extrapolated to zero concentration. The molecular weights of R19-F1A and R19-F2A calculated from the values of $(C/\tau)_{C=0}$ were 900,000 and 1,100,000 respectively. All samples were centrifuged for 30 minutes at 25,000 times gravity to remove optical haze. The refractive index increment for 5461 Å was 0.14 (concentration expressed as weight fraction).

Diffusion. Free diffusion measurements at 2 concentrations of R18-F10 were made at 1.3°C in a Neurath cell using a Longsworth scanning optical system. By the height-area method

$$D^{20°}_{H_2O} = 1.12 \pm 0.11 \cdot 10^{-7}\ \text{cm}^2/\text{sec.}$$

and by the method of successive analysis

$$D^{20°}_{H_2O} = 1.03 \pm 0.07 \cdot 10^{-7}\ \text{cm}^2/\text{sec.}$$

for 5 determinations. Pycnometric measurements gave an apparent specific volume of 0.66 ml/g.

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[*] Present address: Virus Laboratory, University of California, Berkeley, Calif.

[†] Present address, E. I. duPont de Nemours and Company, Box 525, Wilmington, Del.

[‡] We are indebted to Doctors Holzman and Niemann for providing this material, and for helpful suggestions.

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TABLE I. η_{sp}/Φ of A-Substance.

	Average shear gradient of solvent		
$\Phi \cdot 10^3$	10200	5400	1000
R19-F1A			
3.91	150	210	300
1.96	130	170	260
0.78	—	140	240
R19-F2A			
3.31	190	250	400
1.65	170	220	330
0.66	170	220	290

Viscosity. The values of specific viscosity, η_{sp}, divided by volume fraction of solute, Φ, at different rates of shear are given in Table I. Measurements were made in an Ostwald-Fenske viscometer at 25°C.

Osmotic pressure. Four measurements at one and 2% concentration gave the molecular weight of R18-F10 as 220,000 ± 30,000, uncorrected for deviations from ideal behavior.

Discussion. If the molecules are assumed to be anhydrous rod-like ellipsoids, the viscosity data lead to a value of 60/1 for the axial ratio. This value, together with diffusion and density data, gives an average value of 1,000,000 for the molecular weight.

Table I shows the marked dependence of the value of η_{sp}/Φ on shear gradient and on concentration. This behavior is more like that of a rod-like particle than a random coil.§

The molecular weights from light scattering data agree fairly well with that from viscosity and diffusion. Osmotic pressure data lead to a much lower value. Since the latter method gives a number-average molecular weight and the light-scattering method gives a weight-average molecular weight, the results are compatible with the assumption that the material is heterogeneous. The data do not permit one to decide whether a few per cent of larger or smaller molecules account for the results, or the preparations contain a broad distribution of sizes with significant numbers of molecules with molecular weights less than 200,000 and greater than one million.

It may be *concluded* that A-substance from hog gastric mucin, like A-substance from pseudomucinous ovarian cyst fluid, is a very large molecule and probably is highly asymmetric.

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§ We are indebted to Dr. Hans Kuhn for interpreting these data.

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Experimental Bacterial Endocarditis in Altitude Rats II. Shortening Acclimatization Period. (19150)

BENJAMIN HIGHMAN AND PAUL D. ALTLAND. (Introduced by W. F. von Oettingen.)

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, Federal Security Agency, Bethesda, Md.

The mortality rate of subacute bacterial endocarditis, despite antibiotic therapy, averages about 30%(1). Lack of a suitable experimental animal has handicapped hitherto the search for more effective treatment. The screening of therapeutic methods calls for a readily available small laboratory test animal which is highly susceptible to experimentally induced bacterial endocarditis. Since the therapeutic response in man often varies considerably with the specific infectious agent, the experimental animal should be susceptible to various strains obtained from human cases of bacterial endocarditis.

It was shown previously that exposure of rats 4 hours daily for 3 to 6 months in a low pressure chamber to simulated altitudes of 25,000 feet (7620 meters, bar. p. 282 mm Hg) damages the cardiac valves(2), and that ex-

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TABLE I. Mortality Rate, Hematocrit Values, and Incidence of Bacterial Endocarditis in Rats given 8 Intravenous Injections of *Streptococcus mitis,* JH-26, in 12 Days. The Rats Were Exposed to 25000 Feet for Varying Periods before Beginning the Injections at Age 130 Days.

Group	Altitude exposure before inj. (days)	No. of rats	No. died	Mean hematocrit value†		Endocarditis	
				Before inj.	After inj.	No.	%
1.	30	23	12	78.0 ± 1.5	73.4 ± 1.4‡	20	87
2.	30*	16	4		57.8 ± 2.8	7	44
3.	20	16	9	76.5 ± 1.4	71.2 ± 1.1‡	11	69
4.	10	17	4	65.2 ± 1.8	65.8 ± 2.5	8	47
5.	0	11	4		55.9 ± 1.9	5	45
6.	0*	24	7		42.4 ± 0.7	4	17

* Groups 2 and 6 not exposed to altitude after beginning bacterial inj.

† Numbers indicate mean hematocrit values ± the stand. error of the mean. Hematocrit determinations made using 4 to 10 animals per group, immediately before beginning injections and again immediately before autopsy.

‡ Drop in mean hematocrit value after injections is statistically significant. Since group 4 was exposed to altitude during the injection period, the hematocrit value, had no injections been given, should have exceeded 76.5.

perimental bacterial endocarditis could be readily induced in such altitude-exposed rats by the intravenous injection of streptococci (3). Because of the remarkably high incidence of endocarditis produced in such rats with various strains of streptococci isolated from human cases of subacute bacterial endocarditis, and because the rat is particularly suitable for large scale laboratory testing, the method was considered valuable for studies aimed at improving the treatment of bacterial endocarditis. To facilitate such studies, however, an effort was made to reduce the number of bacterial injections and to shorten the period of acclimatization to altitude, and thereby reduce the labor entailed in exposing animals to altitudes over a long period. In this paper we shall show that a high incidence of endocarditis may be induced in altitude rats even when the acclimatization period is less than 30 days. An attempt was also made to correlate the susceptibility to endocarditis with the severity of the polycythemia.

Materials and methods. The methods used in exposing the animals to altitude and in preparing and injecting the bacteria were essentially similar to those described previously(2,3). A series of Sprague-Dawley rats was maintained at ground level (about 340 feet, 104 meters) until 100 days of age and then divided into groups of 11 to 24 animals each. The exposures to altitude (25,000 feet) were begun at staggered intervals so that at 130 days of age, when the first bacterial injection was given, one group each had been exposed 10 and 20 days and 2 groups each 0 and 30 days. During the following 12 days the rats in all groups received 8 intravenous injections, each consisting of 0.5 cc (500 to 800 million organisms) of a 6-hour culture of *Streptococcus mitis* JH-26 in beef heart infusion broth containing 0.5% dextrose. This organism was originally isolated from a case of subacute bacterial endocarditis and was selected because in the previous study nonaltitude rats were found to be relatively resistant to this streptococcus. Survivors were killed 3 days after the last injection. Only one of each of the 0-day and 30-day groups was exposed to altitude during the injection period. Hematocrit values were determined with Van Allen tubes on 4 to 10 animals per group, and endocarditis was determined by histologic studies as previously described(3).

Results. As shown in Table I, the incidence of endocarditis seems to vary with the mean hematocrit value. The mean hematocrit value after 30 days acclimatization was 78 or approximately the maximum attainable by rats exposed to 25,000 feet(2). The incidence of endocarditis in the 23 rats was 87% after 8 injections compared with 100% reported previously after 12 injections in 8 rats exposed

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to altitude for 3 to 6 months. In the 30-day group the mean hematocrit value dropped to 73.3 (statistically significant) after the injections, probably due to the infection. When the exposures to altitude were discontinued during the injection period, however, the drop in hematocrit value was much greater and the incidence of endocarditis was approximately halved. The incidence of endocarditis in other groups likewise seemed to vary with the mean hematocrit value.

As in the previous study, the animals that developed an endocarditis generally lost considerable weight and nearly all rats that died or were killed after the fourth day from the first injection had renal abscesses. Some showed large bacterial masses in the renal pelvis and extensive necrosis of the renal papillae. Endocarditis was found in nearly all the altitude rats that died including 9 of 10 in the 20-day and 30-day groups that died after only 4 injections. None in these groups died after less than 4 injections. In the previous study, however, endocarditis was found in some animals that died after only a single injection of *S. mitis* or other strains of streptococci, particularly *S. faecalis* Strain E.

To study the incidence of endocarditis after a single injection, therefore, 15 altitude rats (exposed 70 days to 25,000 feet) were given a single intravenous injection of 0.5 cc (800 million organisms) of a 6-hour culture of *S. faecalis* Strain E in beef heart infusion broth containing 0.5% dextrose. Bacterial vegetations were found on the heart valves in 3 of 6 such rats killed 20 hours after the injection, in 2 of 3 killed at 48 hours, and in 3 of 3 each killed at 72 and 96 hours after the injection. Two of the 4 animals without bacterial vegetations showed inflammatory changes in the mitral valves, suggesting that vegetations might have ensued if the animals had not been killed. Although this incidence following a single injection is high, it must be emphasized that the incidence after multiple injections was found to vary considerably with different species of streptococci and to a lesser extent in different groups of animals.

Discussion. Since susceptibility to endocarditis seems to vary with the hematocrit value, it is possible that the acclimatization period might be further shortened by prolonging the daily exposures to altitude to speed the rise in hematocrit value. It is also possible that susceptibility might be induced by ground level hypoxemia or by other methods increasing blood volume(4).

The polycythemia induced by altitude increases the work load on the heart and its valves. That this may be an important factor in increasing the susceptibility to endocarditis is suggested by the work of Lillehei, Bobb and Visscher(4). They reported an increased susceptibility to endocarditis in dogs in which an increase in work load on the heart was accomplished by the creation of large arteriovenous fistulae. That additional factors may be involved, however, is indicated by evidence that exposure to high altitude may lower the resistance to other types of infection. For example, it has been shown recently(5), that CF-1 mice made polycythemic by exposure to high altitude were more susceptible to artificially induced *Salmonella typhimurium* infections than normal or anemic mice.

Summary and conclusion. Sprague-Dawley rats were exposed in a low pressure chamber 4 hours daily 0 to 30 days to a simulated altitude of 25,000 feet (bar. p. 282 mm Hg). During the following 12 days, they were given 8 intravenous injections of *S. mitis*. Bacterial endocarditis was found in 87% of the rats acclimatized 30 days, but in only 44% if the exposures to altitude were discontinued after the first injection. It was found in 17% of rats never exposed to altitude, and in 45% of those beginning exposures to altitude immediately before their first injection. Endocarditis was found in 11 of 15 altitude rats given a single injection of *S. faecalis*. The susceptibility to endocarditis in this study varied with the mean hematocrit value. Since the hematocrit value approaches a maximum in about 30 days, there seems to be little indica-

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4. Lillehei, C. W., Bobb, J. R. R., and Visscher, M. B., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 9.

5. Aero Medical Subcommittee. USAF School of Aviation Medicine and Arctic Aeromedical Laboratory Supplement. Air Force Semi-Annual Progress Report, 1951, v2, Proj. No. 21-35-005.

tion for increasing the preliminary exposures to altitude beyond 30 days. It is suggested that such altitude rats be used for screening of methods for the treatment of bacterial endocarditis in man.

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Endogenous O_2 Uptake of Intact Embryos, Homogenates and Cellular Constituents in Isotonic Sucrose and Ringer.* (19151)

Joseph Hall Bodine, Kiao-Hung Lu, and William West.

From the Zoological Laboratory, State University of Iowa, Iowa City.

In the preparation of homogenates from vertebrate tissues, and especially liver, it has been shown that sucrose makes a more satisfactory suspension medium than isotonic balanced salt solution(1-3). This is especially true when the activity of certain enzyme systems is being studied, for example, succinoxidase. Greater activity has been obtained when homogenates and cellular constituents are suspended in isotonic sucrose. For in-

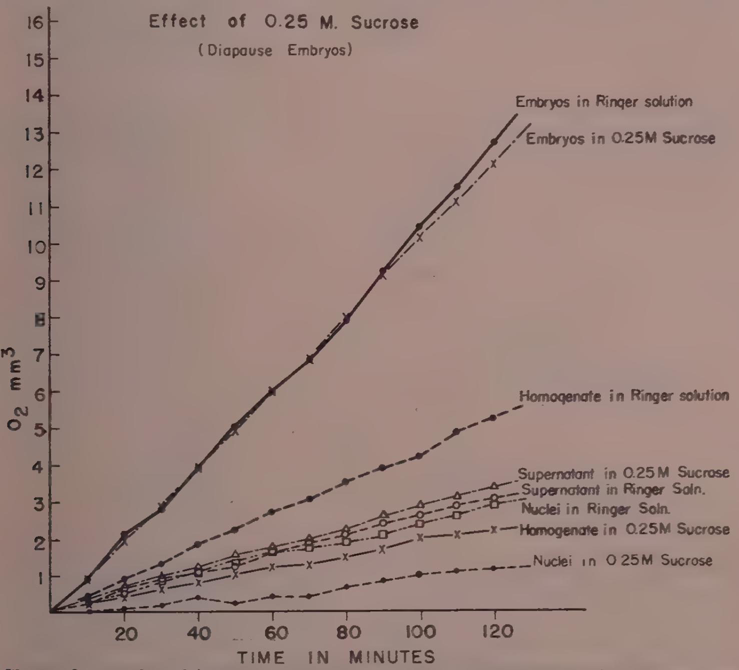

FIG. 1. Shows O_2 uptake of intact embryos, homogenates, supernatant (cytoplasm) and intact nuclei in .25 M sucrose and Ringer's solution. Arrow indicates time of emptying contents of side arm into respiration flask.

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* Aided by a grant from the National Institutes of Health. Acknowledgement is gratefully made to Etta Andrews for technical assistance.

1. Schneider, W. C., Claude, A., and Hogeboom, G. H., *J. Biol. Chem.*, 1948, v172, 451.

2. Lardy, H. A., *Respiratory Enzymes,* Burgess Publ. Co., Minneapolis, 1949, (Contains rather complete bibliography).

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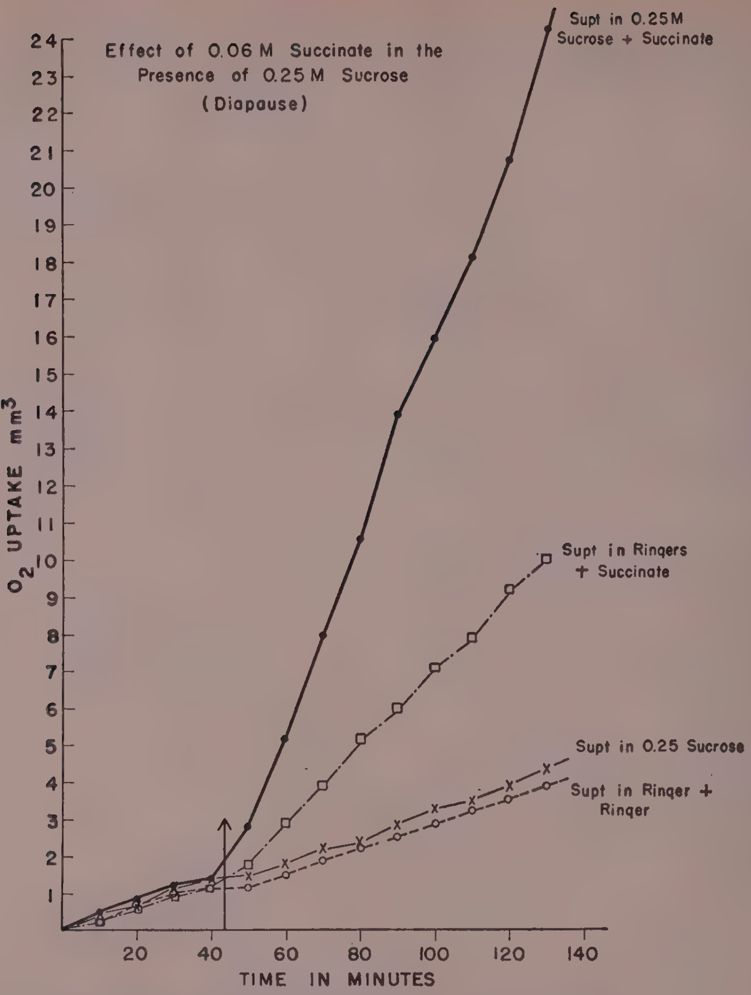

FIG. 2. Shows O_2 uptake of supernatant (cytoplasm) in .25 M sucrose and Ringer's solution with and without addition of .06 M sodium succinate.

vertebrate tissues little such data seem to exist. The present paper, therefore, is concerned with the effects of isotonic sucrose and Ringer solution upon the endogenous O_2 uptake of intact grasshopper embryos, their homogenates and intracellular constituents.

The embryo of the grasshopper, *Melanoplus differentialis,* dissected from the egg and freed of yolk has been used as experimental material. Details of procedure have been repeatedly described and will be omitted here(4). Oxygen uptake was measured with standard Warburg manometers at 25°C using respiration flasks of 5 ml capacity. Sucrose (0.25 M) and Ringer solution, both at pH 6,8 were used as suspension media. One hundred embryos per ml of medium have been used throughout. Homogenization and fractional centrifugation were carried out as previously described(4).

Results of typical experiments are graphically shown in Fig. 1 and 2. Fig. 1 shows that intact diapause embryos consume similar amounts of O_2 in either sucrose or Ringer

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4. Bodine, J. H., and Lu, K., *Physiol. Zool.,* 1951, v24, 120.

over the course of the experiment. Intact embryos homogenized in Ringer solution show a considerably higher O_2 uptake than for similar embryos when homogenized in 0.25 M sucrose. When homogenates are fractionally centrifuged and supernatants (cytoplasm) and precipitates (nuclei) tested, the endogenous O_2 uptake of supernatants whether in sucrose or Ringer is similar. Nuclei suspended in Ringer have much higher O_2 consumption than those in sucrose. It seems reasonable from this to assume that low O_2 uptake for homogenates in sucrose must have been conditioned by the lowered O_2 uptake of the contained nuclei. As a matter of fact, nuclei when suspended in Ringer, appear quite distinct and show no visible signs of injury. On the other hand, nuclei in isotonic sucrose become sticky, clumped and practically impossible to handle with pipettes. They lose their shape, break up and appear as a sticky mass. It would thus seem that nuclei from grasshopper embryos are markedly affected when suspended in isotonic sucrose solution. That other parts of the cell are not so affected by the sucrose seems of interest and especially so when various substrates such as succinate are added to them.

Supernatants (cytoplasm) free of nuclei, and suspended in sucrose and Ringer show rather striking differences in O_2 uptake when sodium succinate is added to them. Results of typical experiments are graphically shown in Fig. 2. An inspection of this figure shows that supernatants suspended either in Ringer or sucrose respire at approximately the same rate. When sodium succinate (0.06 M) is added, however, supernatants in sucrose show increased O_2 uptake, markedly in excess of that shown by those suspended in Ringer. No apparent reasons for this marked difference in response to the succinate are evident. Since succinic dehydrogenase seems to be associated largely with mitochondria it seems reasonable to assume these differences may be due to some effect of the sucrose upon them, possibly a softening or splitting effect making the dehydrogenase more readily available than in the Ringer solution.

Summary. 1. Intact embryos of the grasshopper, *Melanoplus differentialis*, show similar rates of endogenous O_2 uptake when suspended in isotonic, Ringer or sucrose solution. 2. Homogenates of embryos suspended in Ringer solution show much higher O_2 uptake than those suspended in isotonic sucrose solution. 3. Isolated intact nuclei suspended in Ringer solution show much higher O_2 uptake than those suspended in isotonic sucrose solution. 4. Isotonic sugar solution affects the endogenous O_2 uptake of only nuclei and no other parts of the embryonic cell. 5. Supernatants (cytoplasm minus nuclei) suspended in isotonic sucrose show greater stimulation of O_2 uptake by addition of succinate than when suspended in Ringer solution.

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Excretory Function of the Skin in the Squid.* (19152)

BRUNO KISCH.

From the Biological Laboratory, Fordham University, New York and the Marine Biological Laboratories, Woods Hole, Mass.

It is general knowledge that the cephalopoda have a special excretory organ, a kind of nephron for the elimination of waste products. The epidermis of this group of animals contains glands, which in some species eliminate a fluid rich in calcium salts, and in some mollusks produce certain coloring matter. The excretory function of the skin of squids is unknown, therefore the following observations are significant.

One to 4-day-old squids showing pronounced phototropism and aerotropism were

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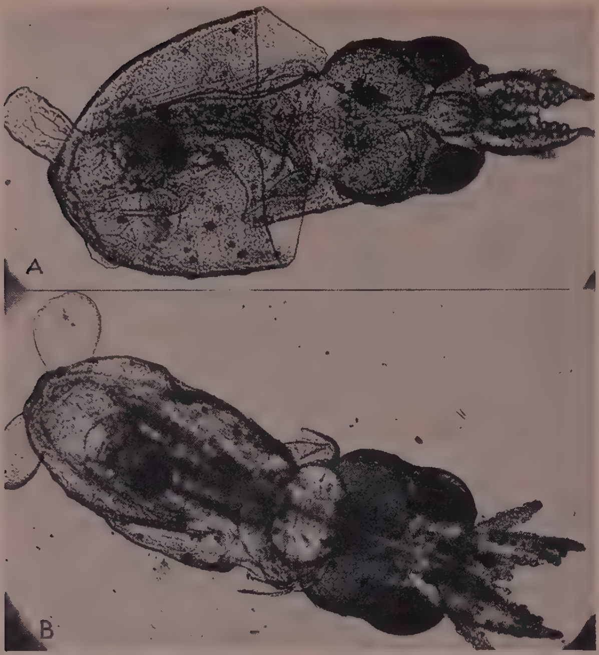

FIG. 1. Squids 2 days old. Slightly colored with neutral red. *Upper figure,* mantle relaxed. *Lower figure,* mantle contracted. Notice the smooth contour of the mantle, no sweat droplets visible. Enlargement 25 ×.

studied. They were made suitable for photographic purposes by adding very small amounts of neutral red to the sea water (0.001%).

Fig. 1A and B show such animals, one with the mantle contracted, one with the mantle relaxed. The surface of the skin appears as a smooth line, only on a few spots of the animal A, a very fine bulging of the surface of the skin, probably indicates places where chromatophores may be present. If the concentration of the neutral red is higher, (0.005% to 0.01%) the animals quickly become very deeply stained and are less satisfactory for photographic purposes. However, a few minutes after the addition of the stain to the sea water, small dark red stained droplets appear first in the apical aboral portion of the animal, but ultimately over the entire skin of the animals. The growth of these little droplets can be observed under the microscope. They become increasingly more distinct, become spherical in shape and finally at a movement of the mantle of the animals, which are swimming around, these droplets separate from the body and being heavier than sea water collect on the bottom of the container. Thus the skin of these animals functions as an excretion organ for neutral red. Apparently this neutral red was combined with mucus or protein, since these droplets do not dissolve in the sea water as neutral red does. Fig. 2A, B, C show the surface of the animals covered with such red droplets. They appear on the mantle skin, on the skin of the head, but not in the region of the eyes.

Summary. The skin of the squid functions as an organ of excretion. This is proven by feeding squids neutral red solution. The neutral red is eliminated by the skin in the form of dark red droplets, heavier than sea water and not soluble in it.

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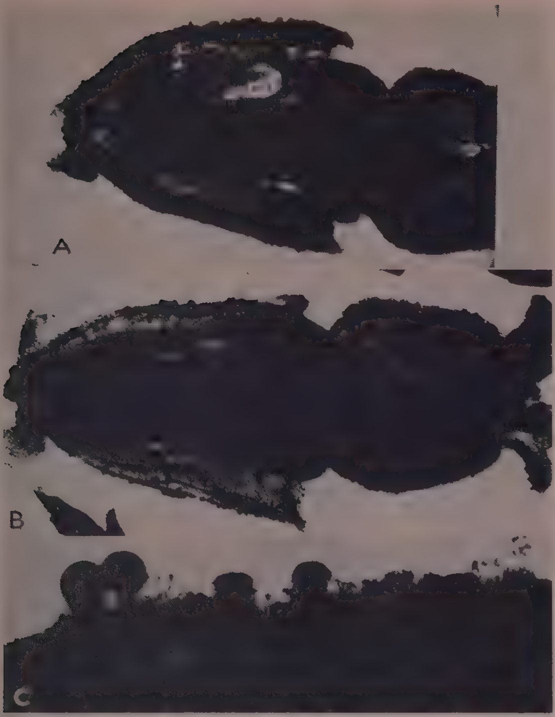

FIG. 2. Two different squids. Age 2 days. Kept for 20 minutes in sea water with a content of .005% neutral red. The surface of the skin is covered with smaller and larger droplets of dark red colored sweat. Enlargement 25 ×. *Lowest figure,* a part of the apical (aboral part of the body) of a squid eliminating droplets stained with neutral red from its skin. (Enlargement 250 ×). Four such droplets well recognizable, growing more and more to a bowl shape. If this stage is reached, slight movement of the animal may separate the droplets from the body.

A Phase Contrast Microscopy Study of X-Ray-Induced Pyknosis in the Living Cell. (19153)

THEODORE N. TAHMISIAN AND DOROTHY M. ADAMSON.
(Introduced by Douglas E. Smith.)

From the Division of Biological and Medical Research, Argonne National Laboratory, Chicago, Ill.

This study was undertaken to determine the cytological effects of X radiation on the living cell. X-ray-induced pyknosis, as seen in fixed preparations, has been discussed in a previous communication(1); however, the effects of fixatives upon these cells were not determined. Therefore, with the aid of the phase contrast microscope, an attempt has been made to determine whether fixatives alter the cytological pattern of irradiated cells. Although the cytological pattern observed in living and fixed preparations appears to be similar, the physical state of the cell components varies in the living material.

Materials and methods. Melanoplus differentialis egg pods were collected daily from an indoor colony and stored on moist sand at a constant temperature of 25° ± 0.02°C for 30

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1. Tahmisian, T. N., and Gasvoda, J., *Quart. Rep., Biol. and Med. Divisions, Argonne National Laboratory,* Aug., Sept., Oct., 1948, eds., A. M. Brues and H. Lisco. ANL-4227, p154-180.

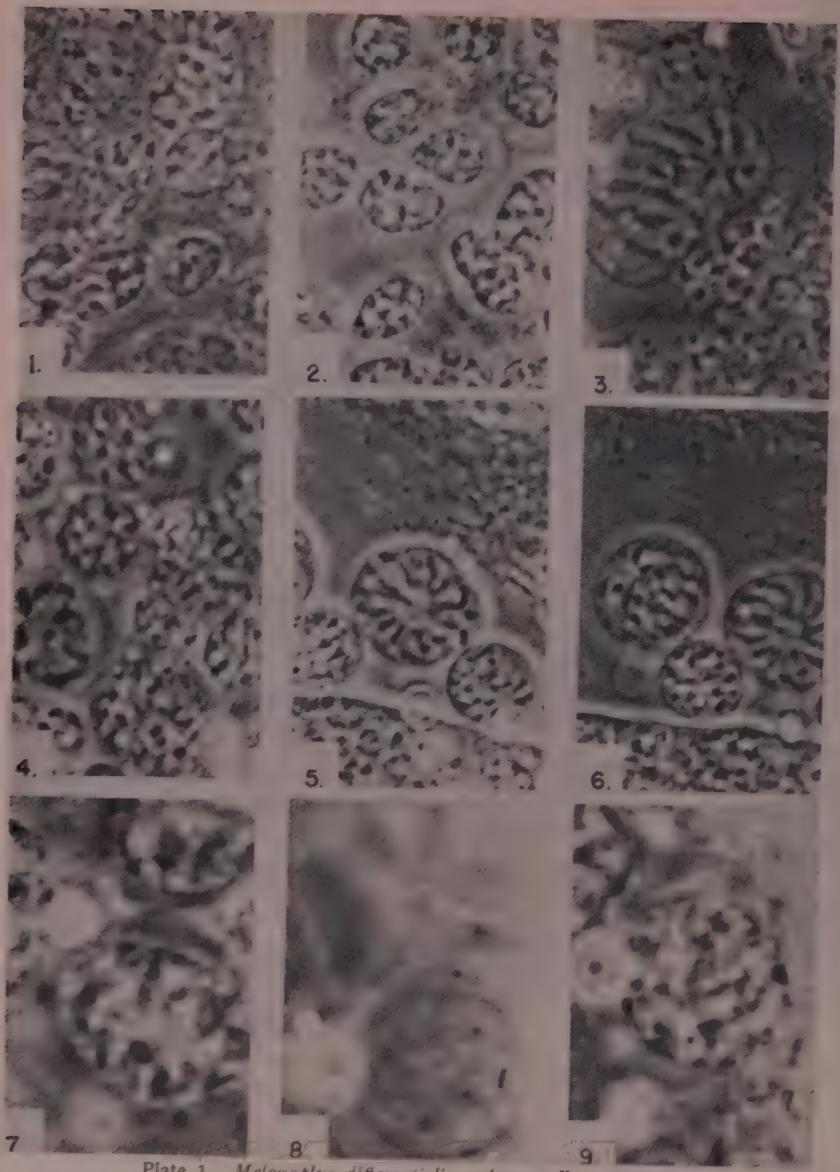

Plate 1. *Melanoplus differentialis* embryo cells. 1120 ×.

FIG. 1. Control, one hr postdiapause.

FIG. 2. Cells X-irradiated with 25000 r 6 mo. previously, kept at 2° ± 1°C. One hr after removal from low temp. 1120 ×.

FIG. 3. Early prophase control, 24 hr postdiapause. 1520 ×.

FIG. 4. Late prophase control, 48 hr postdiapause. 1120 ×.

FIG. 5. Metaphase, polar view, control 96 hr postdiapause. 1120 ×.

FIG. 6. Early anaphase and polar view metaphase, 96 hr postdiapause. 1120 ×.

FIG. 7, 8 and 9. Photographs of the same interphase cell. 1680 ×. (7) In modified Belar sol.; (8) In distilled water; (9) In modified Belar sol. after being placed in distilled water.

days. The eggs were then removed from the pods, washed, sterilized, and dechorionated with 2.5% NaOCl(2). They were rinsed in several changes of double glass-distilled, pyrogen-free water, treated with 0.2% citric acid to neutralize the NaOCl, and rinsed again with distilled water. A Spencer stereo-microscope was used to select the experimental material according to the following criteria: 1) the presence of an embryo, 2) the presence of a cuticle, and 3) an intact amnion which indicated that the egg was in diapause. The selected eggs were divided into two groups; one kept as the control and the other X-irradiated at 25,000 r (dose rate, 1433 r/minute; filter, pyrex petri dish cover; 200 kv; and 15 ma). The irradiated material was divided into two portions, one of which, with appropriate controls, was kept at 25° ± 0.02°C, and the other, with controls, at 2° ± 1°C for 6 months. The embryos were dissected in the following modified Belar solution with final concentrations of 0.9% NaCl, 0.05% KCl, 0.05% $CaCl_2$, and 0.01 M $KH_2PO_4 + Na_2HPO_4$, pH 6.8. The photographs were taken with the aid of a Leitz ortholux microscope that was modified with American Optical Co. phase contrast lenses and a phase condenser. The American Optical Co. dark M phase contrast, 97 X oil immersion with 1.25 N.A., was used. The substage condenser was lowered until a green yellow ring was observed through the telescope, and the pictures were taken on Eastman Kodak 35-mm panatomic X film at a spectral band of 5000 Å to 5800 Å units without other filters. Brownian movement was studied and photographed with the aid of an Eastman Ciné Kodak Special camera at 16 frames per second. The effect of X radiation on the mitochondria of living cells was determined cytochemically with Janus green, (certified vital stain No. D274, City Chemical Corp., N. Y.), 1:50,000 parts in modified Belar solution. The modified Belar solution was used because cells in prophase completed mitosis indicating this solution was not injurious to cells. The effects of Belar, refractive index, 1.3339; distilled water, refractive index, 1.3330; 10% sucrose, refractive index, 1.3480; .5% dextrose, refractive index, 1.4020; buffer (pH 3.40-9.80) at 20°C as determined with the aid of an Abbé Bausch Lomb refractometer; and CH_3COOH and NH_4OH, on the prediapause, diapause, and postdiapause nuclear chromatin were determined (Table I). The condition of chromatin in the nucleus of embryonic cells in their natural fluid and of white cells of undiluted human blood was observed.

Results. In diapause grasshopper eggs irradiation-induced physiological and cytologicals changes can be kept latent for 6 months by maintaining the embryos at 2° ± 1°C. In nature cold treatment is a normal event that breaks the diapause block, and this condition is fulfilled by the onset of winter. If the diapause eggs are not cold-treated for at least one month, no further development takes place. When control embryos are removed from 2° ± 1°C to 25° ± 0.02°C and observed for cytological and morphological changes, they are found to resemble diapause embryos, Fig. 1; however, 24 hours after being placed at 25°C, mitotic figures appear. After 96 hours at this temperature many cells undergo division (Fig. 5 and 6). Eggs that received 25,000 r before a six-month cold treatment period (Fig. 2) showed no irradiation effects during the first three days at 25°C when examined with a phase contrast microscope. On the fourth day irradiation injury became apparent (Plate 2). Of the 379 embryos studied not one normal mitotic figure was found in the irradiated embryos over a period of 18 days at 25°C. Irradiation injury began with a pseudoprophase (Fig. 10-12). The chromosomes became larger and the nuclear membrane became more apparent, but remained intact. As the chromatin showed signs of pyknosis, the embryo began to undergo negative growth(3). At the same time there was an increase in an oxidative system that oxidized hydroquinone at a rate that was 400% higher than in the nonirradiated egg (4). The nucleus in many cells showed an increase in Brownian movement, suggesting that liquefaction was taking place; in others, no motion was apparent indicating gelation. Although the pyknotic bodies appeared morphologically the same in fixed and living material they were physiologically in various

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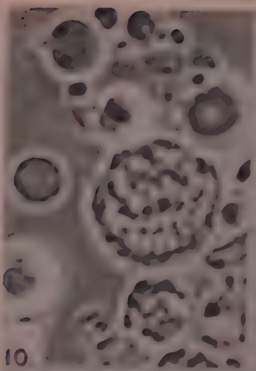
2. Slifer, E. H., *Science,* 1945, v102, 282.

3. Tahmisian, T. N., *J. Exp. Zool.,* 1949, v112, 449.

4. Tahmisian, T. N., and Adamson, D. M., in press.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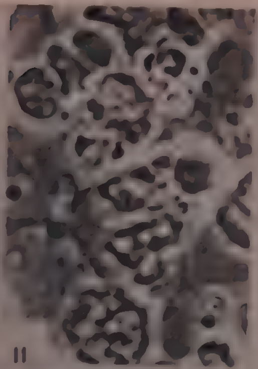
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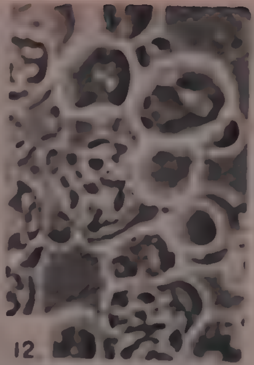
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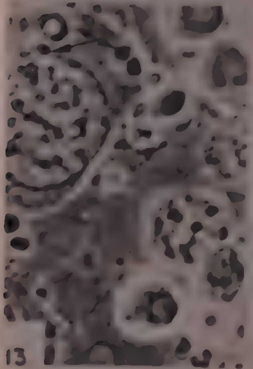
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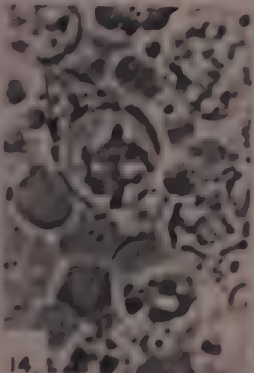
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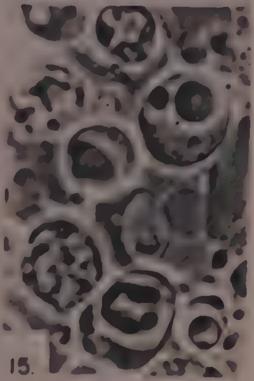
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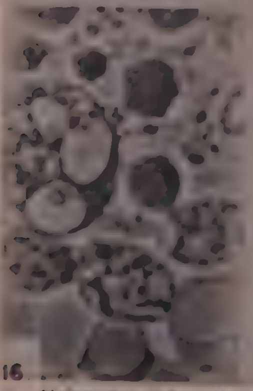
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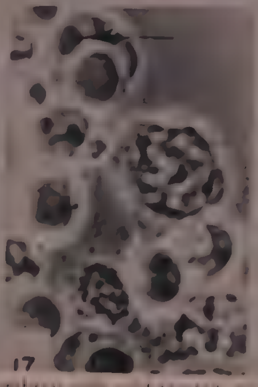
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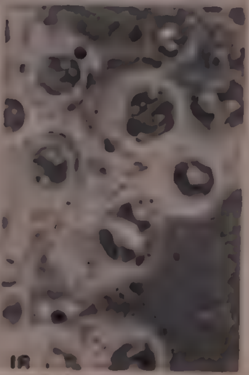
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PLATE [illegible]. *Melanoplus differentialis* embryos were cold treated, 5° ± 1°C, for 6 mo. after X-irradiation with 25000 r, then removed to 25° ± 0.02°C.

FIG. 10. Seventy-two hours after cold treatment, nuclear membrane enlarged, chromosomes sticking to nuclear membrane as well as to one another. 1120 ×.

FIG. 11. Six days after cold treatment. A type of pseudoprophase in the sequence of pyknosis. 1120 ×.

FIG. 12. Six days after cold treatment pyknotic and lacerated nuclei. 1120 ×.

FIG. 13. Eight days after cold treatment. Enlarged nucleus showing incorporation of nuclear membrane with chromatin, also clumping of mitochondria into 3 clusters in cytoplasm. 1120 ×.

FIG. 14. Ten days after cold treatment. Pyknotic chromatin material and large mitochondria at periphery of nuclear membrane. Early segregation of lipoids from the chromatin mass. 1120 ×.

FIG. 15. Twelve days after cold treatment. Lipoid segregation in pyknotic nucleus and absence of lipoid material in pyknotic mitochondrial mass as tested with Janus green. 1120 ×.

FIG. 16, 17, and 18. Eighteen days after cold treatment showing extensive pyknosis and diminution in cell and nuclear size. 1120 ×.

TABLE I.

pH*	Solution	Chromatin	2 min.	On addition of	
3.40 to 9.80	PO_4 Belar	visible	visible	H_2O	invisible
10.50	NH_4OH Belar‡	invisible	invisible	CH_3COOH	visible
6.60	10% sucrose‡	visible	»	Belar	»
6.52	5% dextrose‡	»	»	»	»
6.2	distilled water‡	»	»	»	..

* 6-9.80 the increment was at intervals of .2 to .5 pH.
† These solutions were not buffered.

phases of gel-sol stage in the living condition.

As pyknosis progressed there was always an increase in the size of mitochondria (Fig. 13 and 14). In irradiated cells mitochondria have a high rate of Brownian movement. The rate of movement in the injured cell was much greater than that found in metaphase of normally dividing nonirradiated cells. The mitochondria eventually clumped into a separate body from several small groups (Fig. 13-15). As the chromatin assumed a spherical shape, due to pyknosis, the mitochondria had a similar appearance (Fig. 15 and 17, note arrows). Within the pyknotic chromosomal counterpart lipoid bodies appeared as clear dots under dark medium phase contrast. When cells were fixed in osmic acid-containing fixatives these globules in the pyknotic chromatin were osmophilic(1). This did not hold true for the mitochondrial pyknotic mass (Fig. 15). Eventually the two pyknotic spheres merged and could no longer be identified as separate entities (Fig. 18).

The effects of electrolytes and nonelectrolytes on the living nuclei are shown in Table I. Chromatin was always distinctly visible in the cells of the normal grasshopper embryo when observed with phase contrast microscope. *In situ* studies of the fly *Sciara* show chromatin in the salivary gland(5). Human leucocytes showed the nuclear pattern in undiluted blood if the blood was observed immediately after withdrawal. In the modified Belar solution the cell has the capacity to complete mitosis from a known prophase through telophase. The resting nuclei in the vicinity of the dividing cells showed the chromatin as a distinct phase within the nuclear membrane. The chromatin in an interphase grasshopper cell was distinct in its embryonic fluid under phase contrast in the living condition. If the embryo was dissected in 10% sucrose or 5% dextrose, chromatin structure in the interphase nucleus was present when observations were made within two minutes after dissection. The chromatin in these resting cells lost its identity as the cells remained in nonelectrolytes. If resting cells were dissected in modified Belar solution, the chromatin was distinct. When distilled water was introduced from the side of the coverslip the structure of the chromatin lost its identity. After all nuclear structure had disappeared, addition of modified Belar solution following the water treatment reestablished chromatin structure. Fig. 7, 8, and 9 are a series of phase contrast photographs of the same cell: Fig. 7, in modified Belar; Fig. 8, Belar to which water has been introduced; and Fig. 9, to which in turn modified Belar has been introduced. The refractive indices of the solutions do not indicate loss in intranuclear structure. In distilled water and in 5% dextrose, having the lowest and highest refractions, respectively, the chromatin lost its identity in time. The extended or contracted form of the chromatin in the resting cell does not indicate normality nor injury. In NH_4OH above pH 9.8 no structure was seen, but at pH 3.5 in CH_3COOH acid, the previously lost structure reappeared. Although in both cases the cells were injured.

Discussion. Chromatin structure in the interphase cell of the grasshopper embryo was visible in physiological solution as well as in its own embryonic fluid when observed with dark M phase contrast equipment. The cell had the capacity to complete division from early prophase through telophase in modified Belar solution. The criterion of no injury to cells, as stated by Ris and Mirsky(6), is the capacity of the cell to complete division.

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5. Buck, J. D., and Boche, R. D., *The Collecting Net*, 1938, v13, 201.

We accept this definition and in addition, believe that other cells treated in the same way but which cannot divide will not be injured by the physiological saline in which they are placed. The changes described above in the living cell are attributed by us to the effects of irradiation.

Since a 6-month cold-treatment period following irradiation is known to delay the appearance of injury, changes that were observed in the cell following irradiation are dependent on the metabolic state. If the cell is maintained at developmental temperatures, gross morphological changes take place within a few days following exposure to 25,000 r. This suggests that the cell has lost its capacity to maintain the *status quo*.

Radiation-induced changes consist of an increase in Brownian movement within the cytoplasm as well as in the karyoplasm of most cells. Increase in Brownian movement indicates a drop in the viscosity within the cell following X irradiation at 25,000 r. Other cells, which are relatively few, show increased viscosity as judged by the lack of visible Brownian movement.

The increase in the size of the nucleus and a drop in its viscosity in many cells suggests that a change in the permeability of the nuclear membrane takes place following irradiation. This effect, however, is not immediate but is apparent only after 4 to 8 days following irradiation, when the cell is maintained at metabolic temperatures. In excised nuclei we were not able to detect any osmotic effects upon the membrane. Our method for determining such effects, however, may not be valid since removal of the nucleus from the cytoplasm may have injured it. Irradiated pyknotic nuclei could not be removed from the cytoplasm without the loss of nuclear structure.

The mitochondria in the living, irradiated material showed great Brownian activity and eventually clumped to form clusters in the cells. There seems to be a general rule that the various constituents within a cell that normally are maintained as separate entities do not have the capacity to maintain their spatial relationship for a very long period following X irradiation.

Summary. The effect of X radiation with 25,000 r can be kept latent in *Melanoplus differentialis* embryos for 6 months by maintaining them at submetabolic temperatures. In the resting grasshopper nucleus chromatin, in Belar's saline solution, is visible with the aid of phase contrast microscopy. When the eggs are irradiated and placed immediately at 0°C and kept at this temperature for 6 months no immediate morphological or physiological changes are observed upon returning them to 25°C. However, pyknosis occurs by 8 days at 25°C depending on the irradiation and on the metabolic activity of the cell. Pyknosis in the living cell as observed by phase contrast microscopy resembles fixed material morphologically. Physiologically there is a difference in the physical state of the nuclei when observed in the living condition. Most nuclei show a drop in viscosity at the time of pyknosis as judged by the increase in Brownian movement. Vital staining with Janus green indicates that the mitochondria usually clump as a separate body whereas, the chromatin material becomes pyknotic containing lipoid impregnated bodies.

The authors extend their gratitude to Drs. R. D. Boche of the Institute of Radiobiology and Biophysics, University of Chicago, and A. M. Brues, Director of the Division of Biological and Medical Research, Argonne National Laboratory, for reviewing this paper.

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A Simple Method for Determination of Levels of Amethopterin in Blood and Urine.* (19154)

Joseph H. Burchenal,[†] Georgia B. Waring, Rose Ruth Ellison,[‡] and H. Christine Reilly.

From the Chemotherapy Service, Memorial Cancer Center, and the Division of Experimental Chemotherapy, Sloan-Kettering Institute, New York City.

The marked variation in the amounts of amethopterin or other folic acid antagonists which different patients can tolerate suggests that there may be considerable differences in their ability to absorb and metabolize these compounds. Swendseid *et al.*(1) have used turbidometric methods to study the excretion of aminopterin in leukemic patients. In order to facilitate our studies of the absorption and excretion of amethopterin, a simpler assay method, similar to those used to determine levels of penicillin and streptomycin, was devised. Such a technic is herein described.

Method. Seventy-five g of Difco Folic Acid Assay medium(6), 30 g agar, and 20 μg of crystalline pteroylglutamic acid[§] are added to 2,000 ml of distilled water. This is heated to dissolve the agar, autoclaved 15 minutes at 15 lb pressure, cooled to 45°C in a water bath, and inoculated with 2 ml of a 24-hour-old broth culture of *Streptococcus faecalis* (ATCC No. 8043). After shaking thoroughly to mix the organisms throughout the media, 20 ml aliquots are pipetted into 100 specially pressed, flat bottom Petri dishes (pyrex dishes 100 mm diameter, Corning No. 3162) arranged on a level surface, and allowed to solidify. These plates are then stored in the refrigerator at 4°C and used when desired 1 to 96 hours later. Using a specially purified sample of 4-amino-N^{10}-methyl pteroylglutamic acid (Amethopterin) (7)[¶], 30 standard tubes are prepared containing 5 cc each of a solution of the compound at a concentration of 1,000 mγ/ml. These stock tubes are then frozen at —40°C, and a tube removed every 3 days when new series of standards are required. By diluting this stock solution with distilled water, standards containing 10, 20, 30, 40, 60, 80, 120, and 200 mγ/ml are prepared. These standard solutions are stored in the cold room at 4°C when not in actual use. A standard plate is prepared by placing 4 special filter paper discs, 12.7 mm diameter (Schleicher and Schuell penicillin assay discs), on the agar, and delivering 0.09 ml of one of the various standards to each disc with an automatic penicillin pipette. Since the disc absorbs moisture rapidly from the agar, it is essential that the solution be delivered to the disc immediately after it touches the agar. Several plates are made to cover the various concentration ranges of the standard. The plates are then incubated for 18-24 hours, and the diameter of the zone of inhibition read with a Bermuda Colony Counter (American Optical Co.), containing an etched glass scale graduated in millimeters for measuring zone size. The diameter of the zone is plotted against the logarithm of the concentration of the drug to give a standard curve. Since the concentrations of amethopterin in the urine

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[†] Senior Fellow of the Damon Runyon Memorial Fund for Cancer Research.

[‡] Clinical Research Fellow of the Damon Runyon Memorial Fund for Cancer Research.

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[§] We wish to acknowledge the kindness of the Lederle Laboratories Division of the American Cyanamid Co., in supplying us with this compound.

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[¶] This sample was supplied to us through the kindness of Dr. H. P. Broquist of Lederle Laboratories Division of the American Cyanamid Co.

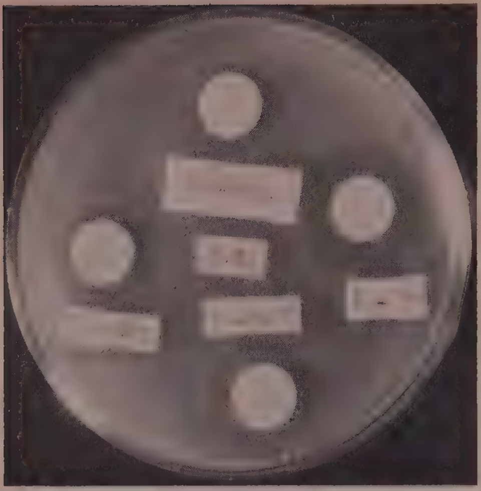

FIG. 1. Urine amethopterin determination with amethopterin standard, 20 mγ/ml, and urine dilutions.

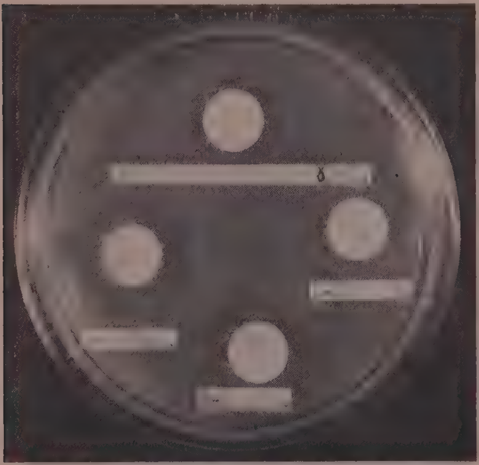

FIG. 2. Serum amethopterin determination.

specimens obtained during a 24-hour period after the oral administration of 5 mg of amethopterin usually range from 1 to 8 γ/ml, considerable dilution is necessary to reach the range (20-40 mγ/ml), at which determinations are most accurate. The usual procedure is to use 4 discs per plate to which are added 0.09 ml portions from a standard amethopterin solution containing 20 mγ/ml, and from 1-500, 1-167, and 1-50 dilutions of urine, respectively (Fig. 1). One to 3 ml samples of blood are collected without anticoagulant at various times after the administration of amethopterin to determine the levels in the serum. The bloods are allowed to clot, rimmed with a wooden applicator stick, centrifuged, and the serum removed. Full strength serum and 1-3 and 1-10 dilutions of this in distilled water are used for most studies. By such dilutions, serum levels of from 10 to 400 mγ/ml can be determined with relative accuracy. As with the urine determination, 4 discs are used per plate, onto which are pipetted 0.09 ml quantities of an amethopterin standard at 20 mγ/ml and of 1-1, 1-3, and 1-10 dilutions of serum, respectively (Fig. 2).

Results. In an attempt to study the recovery of amethopterin, 5 mg were added to 1,000 ml of normal pooled human urine to give a concentration of 5 γ/ml. This was then diluted 1-50, 1-167, and 1-500 and 10 separate plates were set up each containing a standard and all 3 dilutions of urine. A standard plate with concentrations of amethopterin in distilled H_2O was set up at the same time. Recovery values of from 4.8 to 5.6 γ/ml were obtained. On the basis of this experiment, the method would appear to have an accuracy of ±12%. A similar experiment was done in which 1 γ of amethopterin was added to 15 ml of normal serum, giving a concentration of 66.6 mγ/ml, and 1 γ of amethopterin was added to another 10 ml to give a concentration of 100 mγ/ml in the full strength sera. The results on 10 plates of the 1-3 and 1-10 dilutions of the serum containing 100 mγ/ml varied from 81 to 130 mγ/ml and the average of each plate varied from 94 to 111 mγ/ml, giving an accuracy of ±11%. Only the 1-3 dilutions of the serum containing 66.6 mγ/ml were within the satisfactory range for these determinations. With these dilutions on 10 plates, recovery values of 60 to 69.670 mγ/ml were obtained indicating an accuracy of ±10%.

Metabolic studies. Fig. 3 shows the levels of amethopterin in the serum, and the excretion of the drug in the urine after the oral administration of 5 mg of amethopterin. Two normal adult male volunteers in fasting condition (no food for 12-14 hours), and one normal adult female and 3 males, 3, 3, 2 and

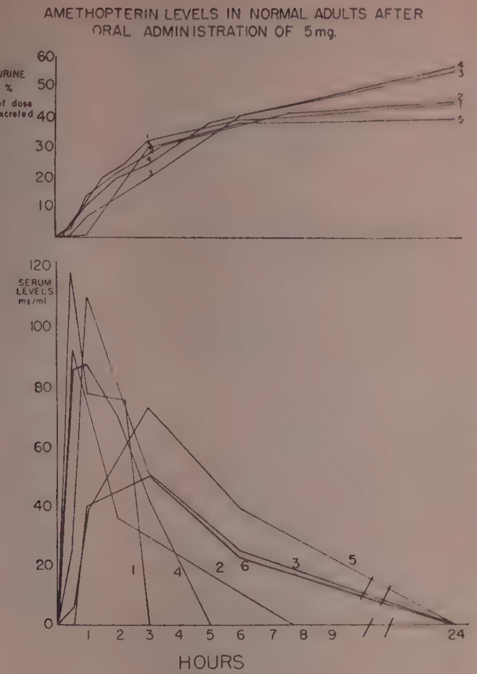

FIG. 3.

2 hours post cibum, were used in these studies. In the fasting adult, serum levels of approximately 100 mγ/ml were attained in the first 30 to 60 minutes after ingestion of the drug, and there was a rapid fall to below detectable levels in from 3 to 7½ hours. In 2 of the individuals (No. 5 and No. 6), who had eaten heavy breakfasts of bread, cheese, and milk 2 hours before the test, there appeared to be delayed absorption and the serum levels rose more slowly, and to a lesser degree. These in turn fell more slowly than in the fasting individuals (No. 1 and No. 2), or in those who had eaten less heartily, or in whom a longer period had elapsed since the last meal (No. 3 and No. 4). In individuals No. 5 and No. 6 a relatively high level was found 6 hours after the administration of the drug, but in none of the 6 volunteers was any detectable amount of the drug present 24 hours after ingestion. Similarly, the urinary excretion of the drug was delayed in No. 5 and No. 6, but the total 24-hour excretion in all individuals was approximately the same, varying from 40 to 57% of the ingested dose.

Serum levels and urinary excretion after 5 mg of amethopterin administered orally on fasting stomach were compared with the values on the same patient obtained after intramuscular administration of the same amount of amethopterin one week later. No significant difference was apparent between the two.

Fig. 4 shows the serum levels after ingestion of 5 mg amethopterin in a fasting male patient with impaired renal function (B.U.N. 58) contrasted with 2 fasting normal males of about the same age. In this patient a serum level of 100 mγ/ml and above persisted for the first 6 hours, and detectable amounts were present in the serum even 48 hours after the ingestion of the drug.

Discussion. A thorough discussion of the advantages and disadvantages of the disc method for antibiotic assay has been given by Loo *et al.*(5). The advantages of this particular technic over the turbidometric tube method for the assay of amethopterin are: (1) that absolute sterility of blood and urine samples is not required, (2) that colored or cloudy solutions or suspensions do not interfere with the accuracy of the test, and (3) that very small blood and urine samples can

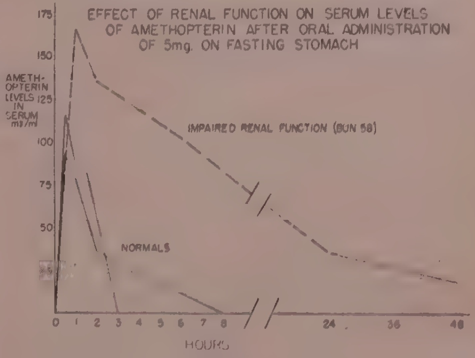

FIG. 4.

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be used if desired, 0.5 ml serum being adequate for duplicate determinations. It is probably true that the accuracy achieved by this technic is somewhat less than that attained by turbidometric assay methods when clear solutions are being tested, but this plate technic compares favorably with similar penicillin assay methods using either discs or cups. Replicate determinations will considerably increase the accuracy of this technic.

In the serum of a patient who has been given amethopterin, all that can be said with certainty at present is that this technic detects a substance with growth inhibitory properties for *Streptococcus faecalis* similar to those of amethopterin. Probably this is the unchanged amethopterin, but it is possible that it may be a metabolite of that drug, rather than amethopterin itself. Chromatographic studies to determine the actual identity of this inhibitory substance are in progress at the present time.

The maintenance of a high level of amethopterin in the serum for the first 6 hours, and the presence of the drug in the serum 24 and 48 hours after ingestion in a patient with impaired renal function, gives a physiologic basis to the clinical finding that relatively small repeated doses of this compound are likely to cause severe toxic manifestations in such patients(8).

Preliminary results here reported suggest that the condition of the stomach (fasting, post cibum) may have considerable effect on the rate of the absorption of ingested amethopterin, but has little effect on eventual absorption as revealed by total 24-hour excretion of the drug.

Although in the one patient studied there was very little difference between the oral (fasting), and the intramuscular routes, it seems probable that for the most accurate metabolic studies an intravenous injection of the drug on a weight basis would be preferable. Metabolic studies by such a technic on patients with acute leukemia are in progress at the present time in the hopes of elucidating the mechanisms of the natural and acquired resistance of this disease to amethopterin.

Summary. (1) A simple disc technic has been devised for determining concentrations of amethopterin in blood and urine. (2) This test has an accuracy which compares favorably with similar assay methods for antibiotics. (3) Amethopterin appeared in the blood and urine in detectable amounts 15 to 30 minutes after the ingestion of a 5 mg dose on a fasting stomach by 2 normal individuals, and 30 to 60 minutes after ingestion at the same dose by 4 normal non-fasting individuals. In some of the 6 it was still detectable at 6 hours, but in none after 24 hours. (4) Six normal adults excreted 40 to 57% of the ingested dose in the urine within 24 hours. (5) In one patient little difference in serum levels or total excretion could be demonstrated whether the drug was given by the oral or intramuscular route. (6) In a patient with impaired renal function, higher initial serum levels were obtained and detectable amounts were present in the blood serum 48 hours after ingestion of amethopterin.

Addendum. Since this paper was submitted, further studies have shown that by decreasing the final concentration of crystalline pteroylglutamic acid in the medium from 10 mγ/ml to 1 mγ/ml a considerable increase in the sensitivity of the technic may be attained.

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Certain Properties of the Causal Agent of Experimental Disseminated Encephalomyelitis in Mice. (19155)

PETER K. OLITSKY AND CHLOE TAL.*

From the Laboratories of The Rockefeller Institute for Medical Research, New York

Acute disseminated encephalomyelitis develops in albino mice after introduction of homologous brain tissue mixed with the Freund adjuvant(1). Not all strains or stocks of mice are, however, susceptible: some are highly resistant, *e.g.*, the Rockefeller Institute strain, and difference in susceptibility exists even between two lines of mice ultimately deriving from the same parent stock, the B-line of W-Swiss strain being relatively resistant while the H-line of the same strain, highly susceptible(2). The availability of such highly susceptible experimental animals offers a proper standard for comparison, and a point of reference for statistical interpretation of the results of the studies to be presented in the present article. The results define properties of the active agent in addition to those already described(1,2), and relate to (a) titration of its concentration in the suspensions as ordinarily prepared; (b) effects of centrifugation; (c) susceptibility of species other than mice to the agent, as well as (d) whether heterologous tissues can also induce the characteristic encephalomyelitis in mice.

Methods and materials. The preparation of materials and the methods used have already been given(1,2). During the course of the present tests the composition of the brain-adjuvant mixture was: Normal brain tissue, 10 g; heavy mineral oil, 50 ml; autoclaved tubercle bacilli, H37Rv type, 250 mg; merthiolate, 20 mg (1:5000 final dilution), and 0.85% NaCl solution, 50 ml, or proportional amounts of this formula as needed. The merthiolate was added to prevent microbic contamination of the mixture which was kept at 4°C; a final dilution of 1:5000 was found satisfactory and in the maximum concentration since 1:500 dilution proved to be toxic for mice (Table I). 0.3 ml of the homogenized material was introduced subcutaneously into H-line Swiss mice. 1 to 3 months old. The standard course of injections in the present series of tests consisted of an inoculation once a week for 6 weeks or until neurological signs appeared. Mice were observed for more than 2 months after the initial exposure. The signs and the pathological picture accompanying the malady have already been described (1).

Titration of the active agent. From an initial suspension of mouse brain in 10^{-1} dilution, further dilutions in the Freund adjuvant were prepared and each was tested in the manner shown in Table I. which also points out the effect of adding merthiolate to the brain-adjuvant mixture. It is clear from this table that when the standard 10^{-1} dilution of mouse brain, with or without merthiolate, is introduced into mice, a uniform reaction is induced. The response is also regular since practically all animals showed characteristic neurological signs or histopathological lesions, or both, after the third inoculation or within a month after the initial exposure. It will also be noted that even after a dilution of 1:1000 was introduced. 5 or 6 mice of 15 developed the experimental disease. Finally, in accord with prior experience(2), an animal exhibited now and again the pathognomic lesions in its central nervous system without any visible sign of neurological disturbance.

Centrifugation tests. 10.5 g mouse brain were homogenized in 42.5 ml saline solution and held at 4°C for 12 hours. The suspension was then centrifuged at 8000 G for one hour. The supernate was removed and kept, and the sediment after resuspension in 10 ml saline solution was recentrifuged. The supernate was added to that of the first centrifugation. The sediment was similarly resuspended and washed 3 times. The wash-fluids were dis-

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* Public Health Research Fellow of the National Institute of Mental Health.

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TABLE I. Titration of Activity of Normal Mouse Brain Suspensions to Which 1:5000 Merthiolate Was Added.

Dilution	No. of mice inj.	No. showing neurological signs	No. showing lesions	Total No. positive	
10^{-1}	35	32	34	34	No merthiolate
10^{-1}	35	35	35	35	Merthiolate 1:5000 added to all others
2×10^{-2}	15	15	15	15	
10^{-2}	15	14	15*	15	
10^{-3}	15	6†	5	5 or 6	
10^{-4}	15	0	0	0	

10^{-5} and 10^{-6} dilutions, like 10^{-4}, completely inactive.

* One mouse showed characteristic lesions but no signs of illness.

† One mouse died, histopathological examination not possible due to postmortem changes.

carded and the sediment resuspended in 52.5 ml saline solution. To the supernate and the sediment respectively were added 50 ml of the Freund adjuvant. Supernate and sediment were homogenized separately in a Waring Blendor and both were injected into mice, 20 for each material. All 20 mice receiving the sediment exhibited characteristic signs of disseminated encephalomyelitis; 1 after the second weekly injection and 19 after the third. These animals also showed the familiar lesions of the experimental disease. All 20 mice receiving the supernate remained well, even after 6 weekly injections and no pathological changes were found in the brains from 2 to 3 months after the initial inoculation. Similar results were obtained on repetition when insignificant changes in the technic were made but always the encephalitogenic factor was found in the sediment, not in the supernate. In another test, a saline-solution homogenate of mouse brain was held for 5 minutes at 100°C in a water bath, and then prepared and centrifuged as above. In this instance the supernate was again inactive in 12 mice but the sediment induced the disorder in 3 of 12 mice; of the 3, two showed no visible neurological signs only the characteristic lesions in the brain.

Heterologous tissues in mice and mouse brain in guinea pigs. The question has not been answered hitherto whether cerebral tissues of other species of animals are active in mice and whether mouse brain is encephalitogenic in another species, for example, the guinea pig which had been shown previously to be highly susceptible to homologous tissue (3). The results would be significant since it is implied that the active agent in mouse brain is of the same nature as that present in other species; hence hetero-reactions, a generality of the latter, should also apply to mouse brain. Guinea-pig and sheep brain were prepared in 10^{-1} dilution in the Freund adjuvant and to the mixture was added 1:5000 merthiolate. The method of preparation was precisely similar to that already described for mouse brain. Guinea pigs received 0.25 ml of the mixture subcutaneously at weekly intervals; otherwise the treatment of the animals was as stated in another section of this paper.

It will be observed from Table II, which summarizes the experiment, that heterologous cerebral tissue was active in mice and conversely mouse brain was encephalitogenic in the guinea pig. The number of reactors and the intensity of effect was not as marked as among mice receiving homologous tissue. This is taken as an indication of a quantitative difference: less of the agent was present in the heterologous brain. The smaller amounts were then accompanied by a suppression of outward neurological signs of the disorder (v. line 3, Table II) at a time when characteristic lesions, sometimes extensive, could be found in the central nervous system.

Discussion. It is apparent from the foregoing results that the merthiolated, 10% suspension of mouse-brain tissue plus adjuvant, which brought about uniformly in mice acute disseminated encephalomyelitis, contained an excess of the active agent. The excess in an ordinary suspension over the

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TABLE II. Effect of Heterologous Brain in Mice and Mouse Brain in Heterologous Species.

Brain from	Injected 10^{-1} dilution into	No. inj.	No. showing neurological signs	No. with lesions	Total No. pos.
Guinea pig	Guinea pig (control)	6	4	4	4
	Mice	20	15	12*	15
		20	0†	13	13
Mouse	Mice (control)	20	19	20	20
	Guinea pig	3	1	1	1
Sheep	Mice	20	8	7*	8

* Others were not examined for lesions; their symptoms were, however, characteristic.

† Indefinite signs were observed such as ruffled fur and sometimes slowness, but no definite neurological signs such as tremor, ataxia, convulsive movements and paralysis.

minimal amount needed to induce the experimental affection was from 10- to 100-fold. Even a dilution of 1:1000 sufficed to produce the neurological disorder in about one-third the number of animals receiving it. In this latter regard Alvord(4) states that guinea-pig brain diluted 1:100 was active in 1 of 3 guinea pigs and 1:1000 inactive in 5, and Lumsden(5) reports that 0.03 g of such a tissue was encephalitogenic in guinea pigs. Thus mouse brain tested in mice offers here an advantage for experimental investigations. Another point of interest is the finding that centrifuged suspensions contained the encephalitogenic agent only in the sediment, not in the clarified supernate. The inference deriving therefrom is that (a) the factor is not soluble; or (b) in whatever form it may exist in nature, the agent needs some element, cerebral tissue itself or a component of it, to set up encephalomyelitis in inoculated animals. In additional experiments with 75 mice attempts were made to determine whether the encephalitogenic factor in mouse brain could be dialyzed(6) against distilled water through a bag of Visking seamless cellulose tubing for 10 days at 4°C. The dialysate, as such and lyophilized, was used. These attempts failed.

Since centrifugation served to localize the encephalitogenic factor in the sediment, thus making its detection more certain, a test was conducted with heated tissue. The result pointed to the active agent being relatively thermostable, a finding for mouse brain which agreed with that for monkey brain(7) and for guinea-pig tissue(5).

The assumption that the encephalitogenic factor present in mouse brain is of the same nature as that found in the cerebral tissue of other species(1) was strengthened by the fact that the mouse-brain agent was active in species other than mouse and heterologous brain was active in the mouse. In any event, the experimental disease produced by the encephalitogenic factor was found to be indistinguishable.

It was noted during the course of the present studies that when the experimental affection resulted from inoculation into more resistant individuals, or of smaller quantities of the active agent, the induced malady was characterized by an absence of any outward neurological signs such as tremors, ataxia, convulsive movements and paralysis, and the presence of more or less marked, typical reaction in the tissues of the central nervous system. Since investigators have pointed out certain resemblances between one or another feature of the induced encephalomyelitis and those of human demyelinating disease including multiple sclerosis(8,9), a comparison of the remission or absence of neurological symptoms in both the experimental affection and the human disease is noteworthy.

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Summary. The present study concerns itself with a titration of the encephalitogenic activity of mouse brain which induced acute disseminated encephalomyelitis in mice. In this regard the active agent revealed a potency higher than that hitherto described for the guinea pig receiving homologous tissue. The encephalitogenic factor was found not in the supernate but in the sediment after centrifugation at 8000 G; nor was it detected in a dialysate of mouse-brain tissue. Mouse brain was encephalitogenic in a heterologous species of animal and heterologous tissues were similarly encephalitogenic in the mouse; the disorder thus brought about by either was indistinguishable from that induced in mice by means of mouse brain.

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Observations of Neoprene Casts of Vascular Bed of the Kidney. (19156)

Melvin A. Block, Khalil G. Wakim, and Frank C. Mann.
From the Mayo Foundation, University of Minnesota, Rochester, Minn.

Neoprene is an excellent material for the study of the structure of the renal vascular bed(1). A serious handicap to its use, however, is the fact that, injected *in vivo*, neoprene causes constriction of the vessels and prevents complete filling of the vascular system. Nevertheless, nearly complete casts of the vascular bed of the kidney can be obtained if the renal vessels are not constricted, but are dilated, and are empty of blood. In cases in which casts of the vascular system are obtained under such conditions the only consistently existing vessels which directly communicate between the renal artery and vein are situated in the hilum of the kidney.

We have obtained casts of the entire vascular bed of the renal cortex and a portion of the medulla by injections of neoprene into the aorta or renal artery *in vivo* provided that the renal vessels were rendered incapable of constriction, were dilated, and were emptied of blood by a brief period of ischemia. *In vivo* injections of neoprene or materials of a similar nature caused a marked renal vasoconstriction as indicated grossly by severe blanching of the kidney. Owing to this vasoconstriction, the filling of the vascular bed of the kidney with neoprene was incomplete. However, when the renal artery or the aorta above the origin of the renal arteries was clamped, after a few minutes the arterial bed of the renal cortex would no longer become constricted when neoprene was injected. Neoprene passed through the arterial and venous beds, and flowed out the renal vein. Surface vessels were filled with neoprene. The clamping procedure itself resulted in a transient cortical vasoconstriction which served to empty the cortical vessels of blood. This emptying of vessels was an additional aid in obtaining a more complete cast of the renal vasculature. Thus, although neoprene provides an excellent medium for the study of the structure of the renal vascular bed, it fails to provide accurate information regarding the intrarenal distribution of the circulating blood.

Especially noteworthy were the casts of relatively long vessels near the hilum, which communicated between the large branches of the renal artery and vein and probably were part of the vascular supply to hilar and pelvic tissues (Fig. 1). The exact caliber of the lumen of these vessels was not determined, but they looked small. These vessels had a greater tendency to form plexuses in the dog than in the rat. When the neoprene injected into the renal artery was of a different color than that injected into the vein, the two colors were found to be mixed in these vessels. These were the only direct communications between the renal artery and vein that were found to be constantly present. It was of interest that in several instances neoprene injected *in vivo*

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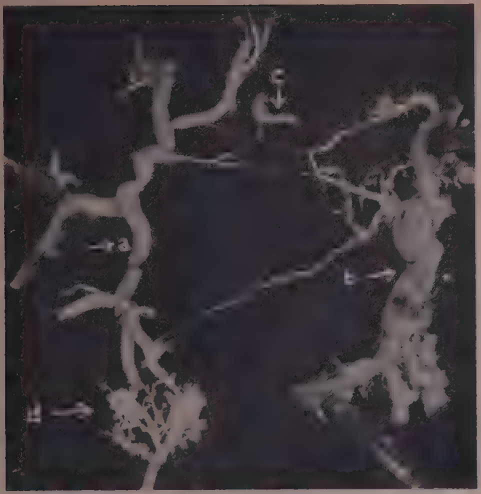

FIG. 1. Communications between arteries and veins in the hilum of the kidney. Injections of white neoprene into the renal artery and of blue neoprene into the renal vein were made into a rat's kidney. A portion of the casts of the hilar vessels were dissected away and mounted separately. One can note the casts of vessels communicating between a large artery, a, and vein, b, in the hilum of the kidney. At c is a short vessel running from one of the communicating vessels to a vessel of blue neoprene, considered to be an interlobar vein. Interlobular arteries with glomeruli from the same kidney are seen at d for comparison of size.

in the rat and dog was found to pass into the renal artery and out the renal vein as seen at the time of the injection, but a cast of only the renal artery and vein and these hilar vessels was obtained. The neoprene had caused a complete constriction of all vessels of the kidney except these hilar vessels, so the neoprene passed directly from the renal artery to the vein through these vessels.

Coiled arterial vessels which communicated between the same or different branches of the renal artery and similar vessels which communicated between the renal vein and its large branches also were found. These vessels were observed by Trueta and associates(2). Spanner(3) reported the finding of arteriovenous shunts in the hilum of one human kidney in which gelatin was injected, but he described short arteriovenous shunts which did not seem to be exactly the same type of vessel as seen in our study.

The results of our study of neoprene casts of the renal vasculature agreed otherwise with observations previously recorded by others indicating that in a normal kidney arteriovenous shunts are extremely rare and are insignificant functionally.

Summary and conclusions. (1) The injection of neoprene into the renal vessels *in vivo* causes vasoconstriction which interferes with the complete filling of the vascular system of the kidney. This vasoconstriction can be prevented by the production of ischemic vasodilatation prior to the use of the injection mass. Satisfactory neoprene casts of the renal vascular bed were obtained by the use of this technic, but the casts do not provide reliable information regarding the intrarenal distribution of the circulating blood. (2) The only consistently existing vessels which directly communicate between the renal artery and vein without an intervening glomerulus are situated in the hilum of the kidney. These vessels are not true arteriovenous shunts but probably are part of the vascular bed of the hilar and pelvic tissues of the kidney.

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The Singularly Brief Anabolic Activity of Testololactone, A Compound Lacking Androgenicity. (19157)

IRVING SHEMANO, GILBERT S. GORDAN, AND EUGENE EISENBERG.

From the Division of Pharmacology and Experimental Therapeutics and the Division of Medicine, University of California School of Medicine, San Francisco.

In the course of studies designed to screen compounds for anabolic activity, with the hope of finding potent anabolic agents with little or no androgenicity, it has previously been shown that anabolic activity does not necessarily parallel androgenic potency(1). The anabolic activity of testololactone,* a nonandrogenic steroid derivative, provides the basis for this report.

Methods. The screening test for anabolic activity was growth of the levator ani muscle of the castrate immature male rat. Testololactone, in a dose of 5 mg per rat per day, produced levator ani muscles weighing 45 ± 1.0 mg in the standard screening test previously described(1). In control animals, the levator ani muscles weighed 33 ± 2.9 mg. To test further the anabolic potential of this compound, its effect upon urinary nitrogen excretion was carried out by the method previously described(2,3). Two such studies were conducted, one using 6 castrate male rats and the other, 5 "plateaued" adult female rats of the Long-Evans strain. These animals were treated with testololactone (10 mg per rat per day). An additional group of 6 castrate male rats was subjected to similar treatment in order to determine the effects of a smaller dose of testololactone (5 mg per day). After the animals were decapitated at the end of the period of administration, the levator ani muscles and seminal vesicles of the castrate males were dissected and weighed by the methods described elsewhere(1).

Experimental data. The data relating to urinary nitrogen excretion are graphed in Fig. 1, and the weights of the levator ani muscles are reported in Table I. Body weight was not significantly altered. It will be noted that in all cases, testololactone produced a moderate but evanescent decrease in urinary nitrogen excretion, amounting at the time of peak effect to 32 to 42 mg per rat per day ($p < 0.01$). Despite continued administration of the agent, the animals did not show continued retention of nitrogen after the fifth day of treatment, and the urinary nitrogen content rebounded to pretreatment levels.

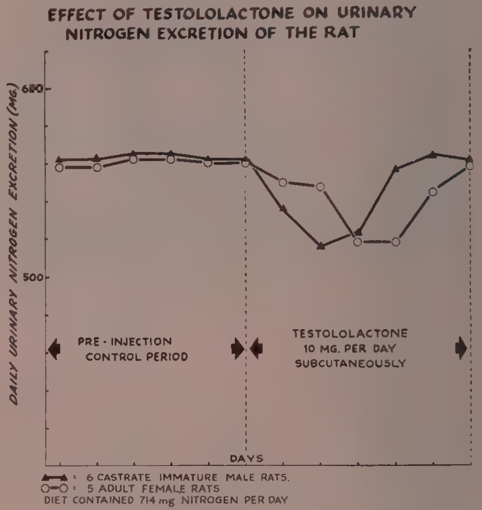

FIG. 1.

Based on the assumption of complete conversion of the retained nitrogen to protoplasm, an increase of body weight of not more than 2 or 3 g would be anticipated in view of the short duration of the period of nitrogen retention. Such changes in body weight are not assessable in rapidly growing castrate males, nor even in relatively "plateaued" adult females.

In contrast to the lack of change of general body weight, there was a statistically significant increase in the weight of one tissue, the

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* Generously furnished by Dr. Irwin Winter, G. D. Searle & Co., Chicago, Ill.

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TABLE I. Effect of Testololactone on Levator Ani Muscle and Seminal Vesicle of Immature Male Castrate Rat.

No. of rats	Group	Levator ani wt, mg ± 6 m	Seminal vesicle wt, mg ± 6 m
6	Control castrates	20 ± 2.1	8.5 ± 1
5	Testololactone, 5 mg/day	31 ± 2.7	8 ± .8
6	Testololactone, 10 mg/day	38 ± 2.2	12.5 ± 2.7

levator ani muscle. The weight of this muscle in untreated and treated animals in this experiment in which dietary restriction was imposed is less than has previously been noted for untreated and treated animals, respectively (1) on *ad lib.* dietary intake.

Androgenic activity, as judged by the weight of the seminal vesicles, was completely lacking, despite the relatively large amounts of testololactone administered for periods of 7 to 9 days.

Discussion. It is typical of the protein anabolic steroids that a retention of nitrogen is produced, followed by an eventual wearing off of the effect or "rebound"(4). Testololactone manifests the most rapid wearing-off of effect of any protein-anabolic compound reported so far.

Kochakian in 1950(4) attributed the "wearing-off effect" of testosterone propionate to a loss of body fat and a shift of carcass protein to the continued synthesis of new tissue at other sites in the body, especially the accessory sex organs. In the case of testololactone, however, such a mechanism is most improbable. Since the effect noted was brief and the rebound during the administration period was rapid, it seems unlikely that the rat would exhaust its body fat in so short a time. Also, testololactone produces no androgenic activity at all under the conditions of this experiment, so a shift of carcass protein to the accessory sex organs cannot be invoked to account for the wearing-off effect of the compound.

Alternative hypotheses for the wearing-off effect immediately suggest themselves. For example, an adaptive process may be taking place so as to activate an enzyme system which inactivates the compound. It is known from studies *in vitro* that enzymatic adaptation may take place in bacteria within a matter of hours. Thus, it is feasible that the wearing-off effect could take place in as short a space of time as observed in this experiment.

The steric configuration of the D ring of testololactone is as yet unknown(5). Inasmuch as all steroids with protein-anabolic activity studied so far have polar groups both at the 3 and 17 positions, it may be suggested that the ketone group of the D ring is likewise in the 17 position. In this connection, we have noted that two other steroids with ketone groups in the 3 and 17 positions produce significant growth of the levator ani muscle without increase in the weight of the seminal vesicles. These are androstadiene Δ 1-2, 4-5 dione 3, 17 and androstadiene Δ 4-5, 6-7 dione 3, 17.[†]

Summary. Testololactone has been demonstrated to have protein-anabolic activity, having passed both the levator ani screening test and a confirmatory nitrogen balance study. This compound showed a rapid effect, remarkably evanescent, which after quickly wearing off was followed by the rebound of nitrogen excretion in the urine to the basal level when testololactone treatment was continued. Testololactone is completely nonandrogenic at the very high dose of 10 mg per day.

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Myofilaments from Smooth Muscle.* (19158)

HASKELL J. WEINSTEIN AND PAUL H. RALPH. (Introduced by H. Stanley Bennett.)
From the Department of Anatomy, University of Washington, Seattle.

Myofilaments with a 400 Ångstrom period have been described in skeletal muscle(1-3) and in cardiac muscle(4). This paper is a preliminary report of the finding in smooth muscle of filaments resembling those of cardiac and skeletal muscle with respect to dimensions and periodic structure.

Method. Five millimeter wide rings were cut from the small intestine of freshly killed turtles and chickens, inverted, and stretched to their approximate maximum physiologic length. The mucosa was stripped off and the muscle rings were fixed in 10% neutral formalin or frozen rapidly in isopentane chilled in liquid nitrogen. The frozen specimens were then dehydrated for two weeks in a vacuum cryostat at –65°C. The formalin fixed material was washed 24 hours in running tap water. Small portions of muscle treated by either method were suspended in water and fragmented with a micro-blender. The resulting suspension was examined with a phase microscope and found to consist of fragments and groups of smooth muscle cells mixed with a small amount of connective tissue fibers and unrecognizable material. Irrelevant material was removed by differential centrifugation and drops of the final suspension were placed on Formvar or collodion films on standard electron microscope grids. The preparations were air dried and shadowed, usually with nickel, and examined with an electron microscope (RCA EMU-2C).

Observations. Fragments of smooth muscle fibers could readily be identified at low magnification upon visual inspection of the fluorescent screen. Figure 1 is an electron micrograph of such a fragment. It reveals the presence of fine filaments extending parallel to the long axis of the muscle cell (B). They range from 150 Å to 230 Å in diameter and appear to be embedded in extrafilamentary muscular substance which appears homogeneous at low magnifications.

Electron micrographs taken at a higher magnification show a definite periodicity that can be readily made out in many of these. The periods range from 322 Å to 743 Å. However, about 90% of all the measurements range from 322 Å to 550 Å. The average period length is 448 Å with a median of 432 Å.

In Fig. 1 there may be seen a number of coarser fibers of a different nature, many of which run crossways to the primary axis of the muscle fragment (A). These are identified as collagen and distinguished from myofilaments by their relatively greater thickness (diameter 400 Å to 550 Å) and their consistently greater longitudinal periodicity (average 660 Å). In many of them the subbanding and polarized nature typical of collagen(5) can be recognized. However, it is not always easy to distinguish myofilaments from collagen filaments, especially in the range where the observed measurements overlap. Similar filaments (Fig. 2 and 3) may be seen in fragmented formalin fixed chicken gizzard.

No clear cut myofibrils, such as readily break out on fragmentation of skeletal or cardiac muscle, have been noted in the present smooth muscle material. Fibrils resembling the "paramyosin" of clam muscle(1,6) have not been observed.

Summary. Myofilaments have been found in the smooth muscle of the gut of the turtle

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* This work was supported in part by grants from the Life Insurance Medical Research Fund, the Eli Lilly Company, the Biological and Medical Research Fund of the State of Washington, and the National Cancer Institute of the National Institutes of Health, Public Health Service.

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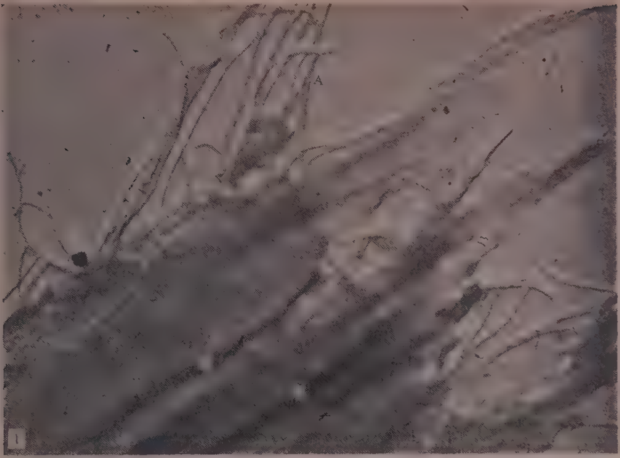
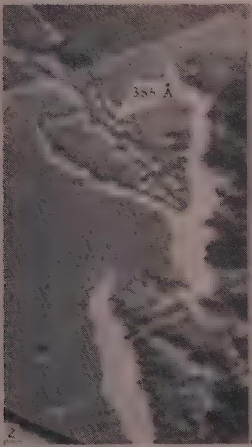


FIG. 1. Electron micrograph of a portion of a smooth muscle fiber from the gut of a turtle, freeze dried, and shadowed with nickel at an angle of 4:1. The greater number of fibers extending to the right and left of the central portion at 'A' are collagen, whereas groups of fibers at 'B' are myofilaments. Darkened longitudinal ridges at 'C' may constitute bundles of filaments embedded in extrafilamentary material and they are visible in fixed material with phase and ordinary bright-field microscopes and would be referred to as smooth muscle myofibrils. Collodion film. 10800 ×.



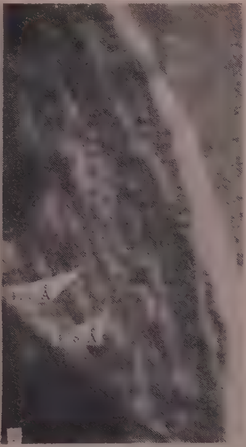


FIG. 2. Electron micrograph of the broken end of a smooth muscle fiber from a chicken gizzard. Formalin fixation, shadowed with nickel at an angle of 6:1. Collodion film. 24200 ×.

FIG. 3. Electron micrograph of a thin sheet of fibers from smooth muscle cell in chicken gizzard. Formalin fixation, shadowed with nickel at an angle of 4:1. Collodion film. 24200 ×.

and chicken. They resemble closely the corresponding filaments of skeletal and cardiac muscle, revealing a repeating period of about 400 Å, and ranging in diameter from 150 Å to 230 Å. Interfilamentary material is also observed in smooth muscle; but is fairly evenly distributed, and not disposed in repeating bands, as in striated muscle.

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Studies on Pituitary Adrenocorticotropin. II. Paper Chromatography of Pepsin Treated Materials. (19159)

W. F. White, W. L. Fierce, and J. B. Lesh. (Introduced by E. E. Hays.)

From the Biochemical Research Department, Research Division, Armour and Co., Chicago, Ill.

Early work with paper chromatography of ACTH fractions in our laboratory(1) and similar work by Payne *et al.*(2) did not indicate any correlation between physiological activity and ninhydrin-reactive spots. More recently, however, we found the correlation to be complete and reproducible in fractions produced by peptic hydrolysis.

The reasons for our early difficulties probably include: (1) The heterogeneity of our early samples leading to poor resolution of ninhydrin-reactive spots; (2) Use of insufficient material to produce distinct coloration; (3) Operation under conditions which gave poor recovery of activity, and (4) Use of unsatisfactory technics for developing the ninhydrin color.

Methods. Whatman No. 1 and Whatman No. 4 paper have been used interchangeably with equal success. Most of the chromatograms have been made by the use of the ascending technic(3). All solvents were redistilled before use and those containing peroxides and/or aldehydes were first purified by the technic described by Newton and Abraham(4). Some technical grade solvents, such as sec-butanol, contain peroxides and completely destroy physiological activity on short contact. Purification, however, usually eliminates this difficulty. Because of this apparent susceptibility of ACTH to oxidative destruction, all chromatograms were run in an atmosphere of hydrogen. In order to eliminate the possible effect of heavy metals, all water used in making up chromatographic solutions and eluants was redistilled in pyrex glass and contained four milligrams of 8-hydroxyquinoline per liter. When assays were to be made on portions of the chromatogram, two identical runs were made on parallel 1½ inch sections of paper with a ½ inch section between. After the run was made and the solvent removed, the sections were cut apart and one was developed with ninhydrin(5). The second section was then cut into the desired segments. Each segment was further reduced by cutting into ⅛ inch strips for elution. Elution of activity was accomplished by immersing the paper in N/10 HCl for 15 minutes. Preliminary experiments on pepsin treated material of known potency showed that under these conditions all of the activity and ninhydrin-reactive material was removed from the paper. Since the amounts of material used on paper chromatograms are necessarily small and the eluates are dilute, such fractions were protected from oxidation prior to assay by the addition of a 0.05 M phosphate buffer at pH 7.0 containing the following additives per liter: 1.0 g glycine, 1.0 g cysteine hydrochloride and 4 mg 8-hydroxyquinoline. For application to the paper, all samples were made up at 10 mg/ml in redistilled water. In order to restrict the size of the original spot, only 0.01 ml was added at any one time. With most samples, 200 to 300 μg of material was required to give distinct ninhydrin spots. In such cases, the paper was thoroughly dried by an infra-red lamp between applications.

Starting materials. All samples used in this study were made from whole hog or sheep pituitary glands by the method outlined in a previous paper(1). The steps in this procedure include the initial extraction and fractionation to produce crude material at a potency of one to 3 times the International

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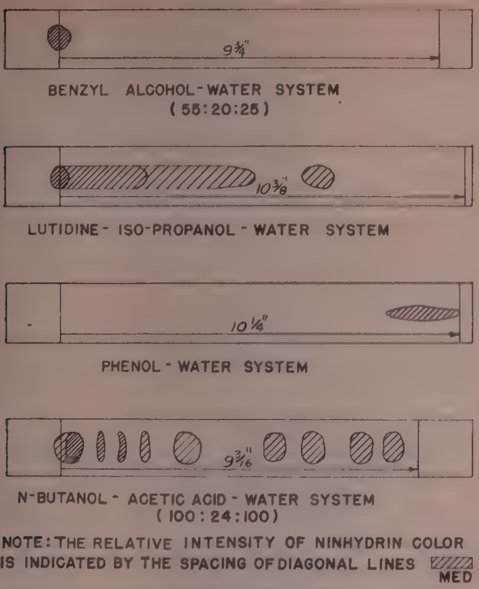
9¾"
BENZYL ALCOHOL-WATER SYSTEM
(55:20:25)
10⅜"
LUTIDINE - ISO-PROPANOL - WATER SYSTEM
10¼"
PHENOL - WATER SYSTEM
9 3/16"
N-BUTANOL - ACETIC ACID - WATER SYSTEM
(100 : 24 : 100)
NOTE: THE RELATIVE INTENSITY OF NINHYDRIN COLOR IS INDICATED BY THE SPACING OF DIAGONAL LINES
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FIG. 1. One dimensional paper chromatograms of pepsin treated ACTH.

ACTH Standard, pH fractionation giving potencies of 5 to 10 times, followed by peptic digestion and trichloroacetic acid extraction giving potencies of 10 to 20 times standard. *Assay.* Physiological activity was determined by the Munson modification of the adrenal ascorbic acid depletion method of Sayers, Sayers, and Woodbury(6). The precision of this assay technic is such that a single determination, using 9 animals at one level compared to the same number of animals on each of 2 levels of standard, is subject to a standard error of 30%. In most of the work reported here, use of duplicates and repetition of experiments has considerably reduced this error. The reference standard used was the International ACTH Standard.*

Results. Preliminary runs (Fig. 1) using both sheep and hog preparations were made with the solvent systems commonly used for amino acids and peptides with the following results: (1) Benzyl alcohol-water. The ninhydrin-reactive material did not move from the point of application. (2) Lutidine-isopropanol-water (55:20:25)(7). Most of the ninhydrin-reactive material remained at the point of application but a somewhat better resolution was obtained than with the benzyl alcohol-water system. (3) Phenol-water. The ninhydrin-reactive material did not form bands but concentrated in a single streamer reaching to the solvent front. Further work with this system has shown that all fractions made from peptic digest material, whether active or inactive biologically give the same pattern. (4) N-butanol-acetic acid-water (100:24:100). Excellent resolution is obtained with this system. Pepsin treated ACTH from hog and sheep pituitary glands give similar patterns. Activity has been recovered only from the area corresponding to the slowest moving ninhydrin-reactive spot. Within the limits of error of the assay, all of the activity placed on the strip was recovered from this area, and no activity was recovered from any other portion. In these experiments the sensitivity of the animal assay was such that 1% of the total activity originally put on the strip was detectable.

In view of the excellent resolution obtained with the butanol-acetic acid-water system, a chromatopile(8) was run using 11 cm Whatman No. 1 paper discs. Three hundred mg of a peptic digest preparation from hog pituitary glands was taken up in water and diluted to 10 ml. Five-tenths ml of this solution was applied to each of 20 discs of the filter paper. The chromatopile was assembled with 40 blank discs at the top, then the 20 containing the sample, and finally 600 more blank discs. Starting at the top, a tagged disc was inserted after each group of 40. Hence, the sample was included in the second group of 40 discs.

The solvent was allowed to flow for 20 hours at room temperature in a confined space and during that time the solvent progressed through about 500 discs. The pile was disassembled, sectioned as tagged, and the discs were air dried. Each section was eluted by

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* Formerly designated as ARMOUR LA-I-A. These assays were carried out in the Biological Control Section with assistance from Mr. J. D. Fisher.

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TABLE I. Biological Activity of Fractions from Chromatopile Treatment of Pepsin Treated ACTH.

Fraction	Wt lyophilized solids (mg)	Original solids, %	Potency (× standard)	Total activity, int. units*	% activity of starting material
S M	300	100	15	4500	100
1	<1	<1	<.5	<.5	<.01
2	24	8	26	624	14
3	25	8.3	56	1400	31
4	20	6.7	34	680	15
5	9	3	<1	<9	<2
Total	78	26		2704	60

* One international unit is the activity of one mg of Int. Standard.

immersing the pack of 40 discs in 100 ml of N/10 HCl and agitating for 15 minutes. The discs were then transferred to a Buchner funnel and with the application of a vacuum were washed, first with the excess eluting solution and then with 100 ml of water. The eluates were adjusted to pH 3.5 with NaOH and concentrated *in vacuo* in a stream of hydrogen to about 25 ml. The concentrates were freed of salt by extracting biologically active material with liquid phenol and then reconstituting it in a fresh aqueous phase by the addition of ether. The resulting aqueous solutions were lyophilized and assayed.

In Table I is shown a summary of the fractions obtained from one such chromatopile in which 300 mg of a peptic digest material with a potency of 15 times the International Standard was used. Concentration of activity in fractions 2, 3, and 4 occurred with 30% of the total activity appearing in fraction 3 at a four-fold increase in potency. Other experiments have given similar results with recoveries of activity up to 100%.

The fractions from the chromatopile were tested by subjecting them to one dimensional paper chomatography. In Fig. 2 is shown the performance of Fractions 2 to 5 in the system n-butanol-acetic acid-water. Fractions 2 and 3 appear free from any ninhydrin-reactive material except that contained in the spot where the activity is concentrated. In view of the higher potency of Fraction 3, most of the subsequent work was done with this fraction. In the systems benzyl alcohol-water, collidine-water, lutidine-isopropanol-water, and methyl cellosolve-water (9:1)(9) Fraction 3 gave only one spot and that at the point of application. Phenol-water again gave a single ninhydrin-reactive area in the form of a streamer reaching to the solvent front.

Discussion. An ACTH fraction has been obtained which is not resolved in the solvent systems commonly used in the separation of amino acids and peptides. The R_f value of this material, however, is very low in all systems except phenol-water. The formation of a long streamer in the phenol-water system is not typical of the low molecular weight peptides reported in the literature(10,11). In fact, the appearance more closely resembles

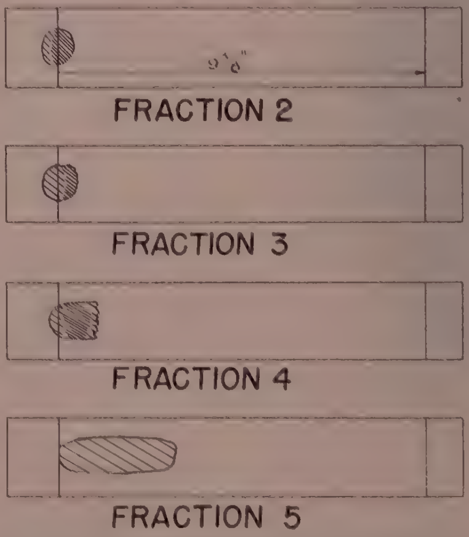

FIG. 2. One dimensional paper chromatograms of fractions from the chromatopile.

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the patterns obtained with proteins(12). This suggests the possibility that the product of the chromatopile has a higher molecular weight than the peptides so far studied in the solvent systems reported in this paper. This possibility is strengthened by the observation that the ninhydrin color produced by Fraction 3 is about one-tenth that obtained from an equal weight of an amino acid. A quantitative determination of ninhydrin coloration by the method of Moore and Stein(13) showed that 1 mg of Fraction 3 gives a color equal to that produced by about 0.06 mg alanine. If a mole-for-mole equivalence of color can be assumed, this would indicate a molecular weight of about 1500 for Fraction 3. Similar determinations on the starting materials for the chromatopile give a value of about 1200. Attempts are being made to devise new systems capable of resolving Fraction 3. Meanwhile, such samples provide excellent starting materials for studying the effects of enzymatic and acid hydrolysis.

Summary. Pepsin-treated ACTH materials at approximately 10 times standard were fractionated in a chromatopile using the system butanol-acetic acid-water. The activity was found to be associated with a slow-moving ninhydrin-positive area from which fractions having potencies of about 50 times standard were recovered. No further chromatographic resolution of these fractions was obtained in the solvent systems ordinarily used for amino acids and peptides. In all cases, the activity was associated with ninhydrin-positive areas of the chromatograms.

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Relationship of Ascorbic Acid to Secretion of Adrenocortical Hormones in Guinea Pigs.* (19160)

Albert B. Eisenstein and Robert E. Shank.

From the Nutrition Laboratory, Department of Preventive Medicine, Washington University School of Medicine, St. Louis, Mo.

Ascorbic acid was first isolated from animal tissues by Szent-Gyorgi who found the vitamin in significant quantities in the adrenal gland (1). Subsequently it was shown that ascorbic acid is present in greater concentration in the adrenal cortex than in any other tissue(2). It had been observed also that the adrenals hypertrophy in scurvy. This change has come to be recognized as one of the pathologic criteria of vit. C deficiency(3-5). These facts have stimulated interest in the relationship of ascorbic acid to adrenocortical function with the result that numerous investigations have dealt with the problem(6-13).

This paper reports the results of a study in which the secretion of adrenocortical hormones was determined in guinea pigs on

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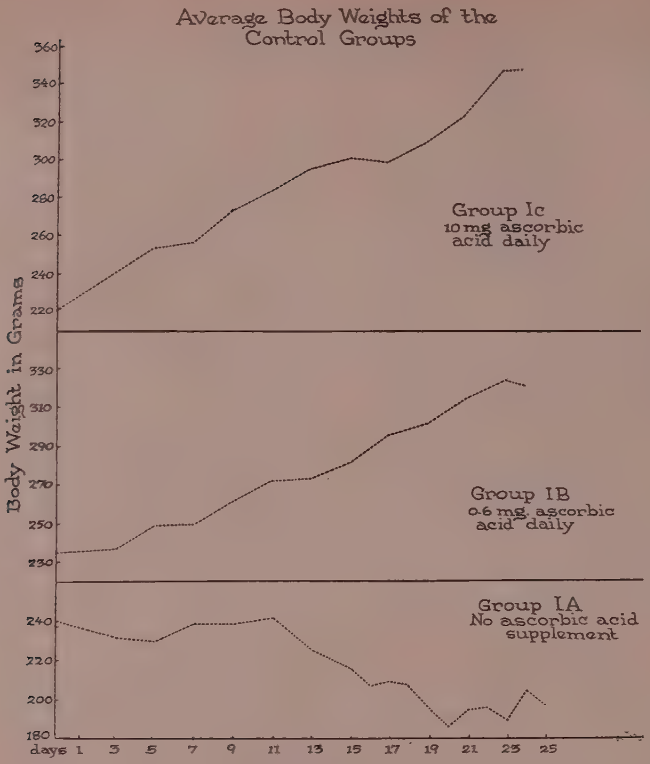

FIG. 1.

graded levels of intake of ascorbic acid. The observations indicate that deficiency of this vitamin functions as do other types of stress but that the vitamin is not directly involved in the elaboration of hormones of this gland.

Procedure. Young male guinea pigs weighing from 200-285 g were used in these experiments. All animals were fed *ad libitum* a scorbutigenic diet described by Zilva(14). One group of 20 guinea pigs was given ACTH while another group of 23 served as controls. Animals in each group were placed in one or another of 3 subgroups which were provided graded supplements of ascorbic acid. The first subgroup (A) received no supplements; each animal in the second (B) was given 0.6 mg of ascorbic acid daily; and those in the third (C) received a daily supplement of 10 mg. These doses were selected so as to provide approximately the minimum daily requirement for the guinea pigs of subgroup B and a more than adequate intake for the animals in subgroup C. The solutions of ascorbic acid were injected intraperitoneally and were prepared immediately before injection by dilution in isotonic saline. Administration of ACTH in the treated group began on the fifteenth day of the scorbutigenic diet. By this time all animals in the group without supplementary ascorbic acid had attained maximum weight and in most instances were beginning to lose weight. The hormone was administered intramuscularly or subcutaneously in quantities of 2 mg per day distributed in 4 equal doses given at intervals of approximately 6 hours. In preparation of ACTH for injection, the hormone was tritu-

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rated in isotonic saline, the solutions adjusted to pH-3 and diluted to the desired volume. This preparation was then frozen and stored until used. Animals were weighed daily throughout the experimental period. The level of circulating eosinophiles was determined at intervals of from 2 to 4 days and served as a measure of adrenal cortical hormone secretion. Eosinophile counts were done by a modification of the method described by Randolph(15). Scorbutic animals were sacrificed by a blow on the head when moribund. When a majority of the animals with scurvy in each group had been killed, the remaining animals in that group were sacrificed. Autopsies were performed immediately after death and the adrenals and a portion of the liver were removed, weighed, and prepared for analysis. Ascorbic acid analyses were done on these tissues by the method of Roe and Keuther(16).

Results. Control group. The variations in growth of the control group during the experimental period are demonstrated in Fig. 1. Guinea pigs which received 0.6 mg of vit. C as a daily supplement (Group IB) gained an average of 97 grams while those given 10 mg of vit. C daily (Group IC) gained an average of 128 g. Animals not receiving ascorbic acid supplements (Group IA) gained weight at a slower rate for eleven days, after which progressive weight loss ensued.

The number of circulating eosinophiles decreased progressively throughout the experiment in 5 of the 8 animals on the ascorbic acid-free regimen. (Fig. 2) The remaining 3 guinea pigs were found at the initiation of the experiment to have low eosinophile counts which remained without significant change. The average eosinophile count at the onset of the experimental period was 127 ± 11.3/mm^3. As severe scurvy developed all counts approached zero and remained at this low level until death. The average count shortly before death was 10.6 ± 3.2/mm^3. Determinations on animals receiving supplements of ascorbic acid were variable. Most had higher levels during the latter part of the experiment than at the start. No animal to which ascorbic acid was administered was found to have progressive decline in the eosinophile count. All animals without supplements of vit. C developed scurvy and survived for 17-25 days with a mean survival period of 20.5 days. Typical gross lesions of scurvy including hemorrhages into joints and intestines as well as adrenal hypertrophy were found. The actual adrenal weights and the adrenal weights in mg per gram of body weight in animals receiving ascorbic acid were significantly less than those of the scorbutic animals. (Shaded areas Fig. 3 and Table I). The concentration of vit. C in the adrenal cortex and liver varied directly with the daily intake of this vitamin.

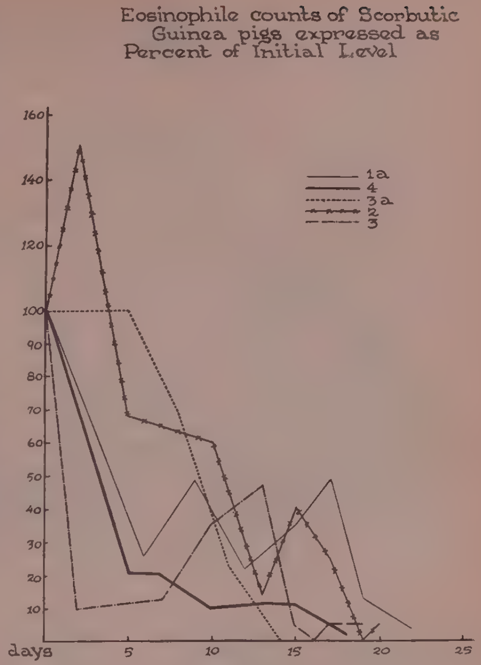

FIG. 2.

ACTH-treated group. The guinea pigs in this group without supplementary ascorbic acid attained maximum weight on the fourteenth day. ACTH administration began one day later. The rapid loss of weight noted in the control animals as scurvy developed was not seen in the treated guinea pigs. Actually 4 of the 7 animals showed cessation of weight

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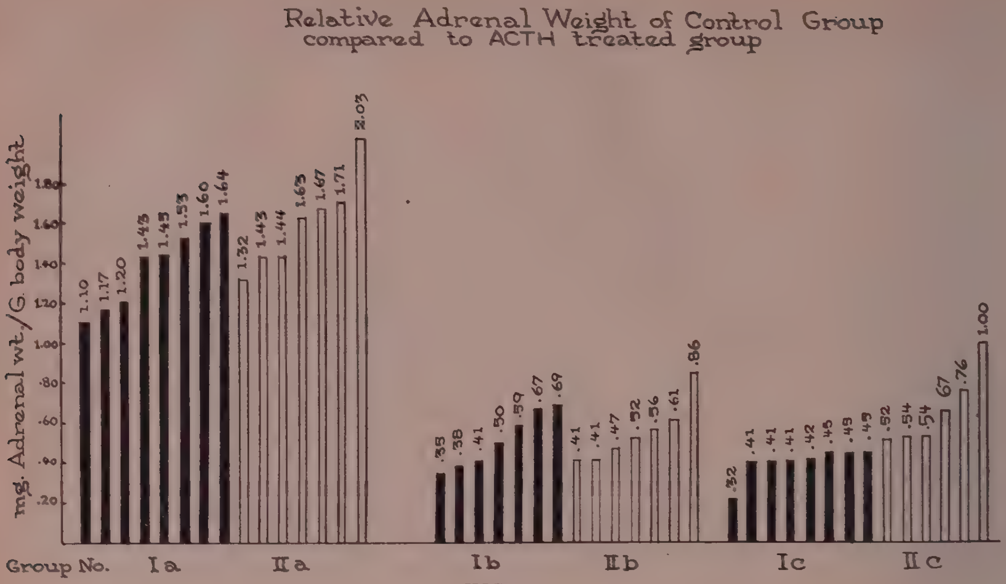

FIG. 3.

loss and for a period of 2 to 5 days again gained weight. Following this secondary peak in weight, progressive loss of body weight ensued and continued until death. (Fig. 4) Of the remaining three animals, two continued to lose weight after ACTH administration began but the weight loss proceeded slowly. The other animal showed a progressive decrease in weight which was seemingly unaffected by ACTH administration.

The number of circulating eosinophiles of this group of animals was variable prior to administration of ACTH. During this period the average number of eosinophiles was 68 ± 8.24 mm^3. However, within two or three days after institution of ACTH therapy, eosinophile counts in all animals fell to lower levels, the average value at this time being 34 ± 5.8/mm^3. Following the initial decrease there was a progressive increase in eosinophile count in guinea pigs which had not received supplements of ascorbic acid. It was considered that this rise might represent evidence of development of adrenal insufficiency. Therefore, to determine if the adrenal cortex retained its ability to respond to the hormone a large additional dose of ACTH (12.5 mg) was administered intramuscularly to 4 of the scorbutic animals. Eosinophile determinations were done prior to and 4 hours after the injection. In each animal there occurred a decrease of more than 50% in the eosinophile count.

Survival of the ACTH treated scorbutic animals was from 26-35 days with a mean survival period of 28.1 days. When these guinea pigs were sacrificed it was found that adrenal hypertrophy was somewhat greater than in the corresponding control animals. Actual and relative adrenal weights of animals on 0.6 mg ascorbic acid daily were approximately the same as those of the corresponding control group while the animals on a daily supplement of 10 mg of ascorbic acid were found to have greater actual and relative adrenal weights than the corresponding control series. (Fig. 3 and Table I).

Concentrations of ascorbic acid in adrenals and livers of these animals varied directly with the intake of vit. C (Table I). The ascorbic acid content of the tissues of guinea pigs receiving 10 mg of vit. C and ACTH were less than the corresponding control animals but this difference was not statistically significant.

Discussion. These observations indicate an inverse relationship between the daily intake of vit. C and adrenal weight. The mean adrenal weight (actual and relative) of the

TABLE I. Adrenal Weights and Concentration of Ascorbic Acid in Adrenal Glands and Liver of Guinea Pigs in Control and ACTH Treated Groups.

Group	No. of animals	Daily supplement of ascorbic acid (mg)	ACTH admin.	Mean adrenal wt ± S.E. (mg)	Mean relative adrenal wt ± S.E. (mg/g body wt)	Mean adrenal ascorbic acid content ± S.E. (mg/100 g)	Mean liver ascorbic acid content ± S.E. (mg/100 g)
I A	8	0	0	246.3 ± 11.75	1.39 ± .096	1.18 ± 44	0
IIA	7	0	+	314.3 ± 12.3	1.6 ± .12	.83	.3
I B	7	.6	0	163.6 ± 16.3	.512 ± .058	19.2 ± 1.8	3.31 ± .64
IIB	7	.6	+	172 ± 10	.55 ± .07	17.5 ± 2.58	2.29 ± .29
I C	8	10	0	144.8 ± 7.6	.409 ± .02	124.2 ± 21.8	19.3 ± 2.53
IIC	6	10	+	211.2 ± 12.8	.67 ± .099	82.8 ± 8.95	14.4 ± 2.18

control guinea pigs which received no supplements of ascorbic acid was much greater than that of control animals receiving supplements. While weights of the glands of animals on 0.6 mg of ascorbic acid daily were greater than those of the group receiving 10 mg each day, this difference was not of sufficient magnitude to be statistically significant. It is conceivable that this latter difference might have been greater had the experimental period been longer. A recent investigation had demonstrated that not until the ascorbic acid-cholesterol ratio in the adrenal cortex falls to below 1:500 does adrenal hypertrophy occur (17). An interpretation of these findings which seems wholly tenable is that a diet deficient in vit. C acts as a form of stress to which the adrenal responds by hypertrophy comparable to that which occurs in other conditions of chronic stress. Administration of ACTH produced adrenal hypertrophy in the animals at the highest level of supplementation of ascorbic acid. Guinea pigs on the completely deficient diet and those on an intake of 0.6 mg of vit. C daily responded to this hormone by somewhat greater adrenal hypertrophy than corresponding controls, but the differences were slight and not of significance. It seems possible that this small dose of ACTH is sufficient to produce adrenal hypertrophy in animals with adequate intake of ascorbic acid but when given to animals already adapting to chronic stress and in which the pituitary is already secreting moderate to large amounts of this hormone, the dose is insufficient to produce further hypertrophy. When it was first noted that ACTH caused a temporary cessation in loss of body weight, this was attributed to fluid retention. However, it soon became apparent that continued administration of this hormone delayed the onset of severe symptoms of scurvy, and moreover the mean survival period of the treated animals was 8 days greater than that of the controls. It is postulated that the temporary alleviation of scurvy resulted from release of ascorbic acid from the adrenals under influence of the hormone. It has been shown by others that ACTH reduces the adrenal cortical content of ascorbic acid(18) and Beck has reported that ACTH administration increased the urinary output of ascorbic acid in an infant with scurvy(19).

The relationship of ascorbic acid to the function of the adrenal cortex has been investigated by many authors with conflicting results. Giroud *et al.*(6-8), Dugal and Therien(20), and Lowenstein and Zwemer(9) have indicated that adrenal cortical function is dependent on an adequate supply of ascorbic acid. On the other hand, Long(10), Jailer (11), Hyman(12), and Treager(13) have accumulated evidence which indicates that this vitamin is of little or no importance in secretion of adrenal cortical hormones.

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20. Dugal, L. P., and Therien, M., *Endocrinology*, 1949, v44, 420.

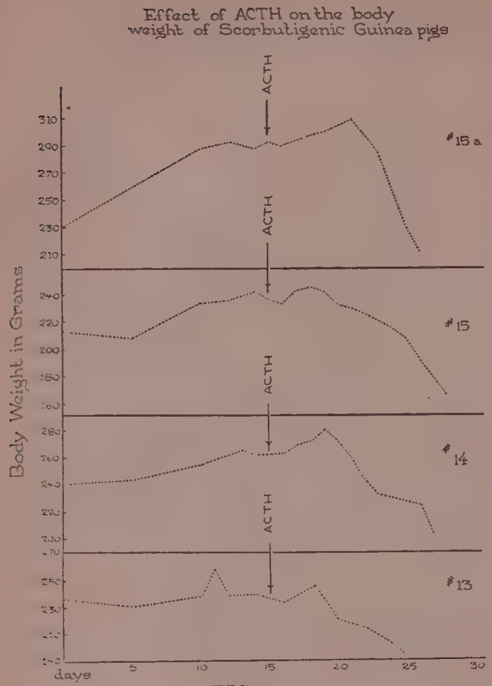

FIG. 4.

A fall in the level of circulating eosinophiles has been shown to be a sensitive measure of the secretion of adrenal hormones with an oxygen atom at the C-11 position(21). The ability of the scorbutic animals in the experiments herein reported to maintain their circulating eosinophiles at very low levels until the time of death may be due to the nutritional deficiency *per se* or may indicate continued production of cortical hormones. When it was thought adrenal insufficiency might be developing in scorbutic animals treated with ACTH, a large additional dose of the hormone was capable of stimulating production of 11-oxysteroids by the adrenal. Thus, the ability of the adrenal to respond to endogenous and exogenous ACTH with secretion of 11-oxysteroids at a time when severe scurvy exists would seem to support the view that ascorbic acid is not essential for the adrenal cortex to secrete hormones of this type.

Summary and conclusions. (1) The weight of the adrenal glands of guinea pigs on graded levels of intake of ascorbic acid varied inversely with daily intake of this vitamin. (2) A progressive decrease in the number of circulating eosinophiles occurred in guinea pigs maintained on a scorbutigenic diet. (3) There was a delay in onset of severe symptoms of scurvy and an increase of the mean survival period in scorbutic animals treated with ACTH. (4) The administration of a large dose of ACTH to guinea pigs with severe scurvy was promptly followed by a decline of more than 50% in the level of circulating eosinophiles. (5) These observations are interpreted as indicating that ascorbic acid deficiency functions as a non-specific stress and that this vitamin is not directly involved in mechanisms of elaboration of adrenal cortical hormones with an oxygen atom at the C-11 position.

The authors wish to express their appreciation to Armour Co. for the ACTH used; to the Abbott Laboratories for supplies of ascorbic acid; and to the Pet Milk Co. for the powdered whole milk used in preparation of the diet.

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Induction of Heritable New Type in Type Specific Strains of *H. influenzae*.* (19161)

HATTIE E. ALEXANDER AND GRACE LEIDY.

From the Babies Hospital and the Department of Pediatrics, Columbia University College of Physicians and Surgeons.

Change in the inherited characteristics of pneumococci(1) and *H. influenzae*(2) induced by highly specific desoxyribonucleic acid containing extracts is now well established. Thus far all reports have been concerned with transformation in R cell populations, with one exception: Taylor(3) has presented data on the induction, by fractions containing desoxyribonucleic acid, of changes in mutants of type 3 pneumococcus which exhibited varying degrees of abnormality of the type 3 factor; the changes involved only homologous type. The present report offers evidence that a change in type specificity can be induced in a fully equipped type specific population of *H. influenzae* derived directly from a single clone.

Methods. The types b and d strains in which new types were induced included one from which R colonies had been used for earlier reports(4). The type b strains had been isolated from the spinal fluid of patients with influenzal meningitis, and the type d strains from the nasopharyngeal mucus of patients with respiratory infections. Soon after growth from these sources, the cultures were desiccated and sealed under vacuum by a method (5) which preserves their original status. The method of preparation of desoxyribonucleic acid extracts, the concentrations used in the transforming environment, and the procedure used were not different from those reported previously(4). The environment used for transformation differed in only one respect, the presence of type specific antibody corresponding to the recipient cell type in place of anti R antibody; a 1:15 or 1:20 dilution of specific antiserum, produced in rabbits during one month of immunization, was employed. In order to aim for a genetically homogeneous culture, the suspension used for transformation was derived from a single S clone (colony).

Results. Three of the 5 type b populations tested were transformed into types a, c and d. Types a, b and c were induced in both of the type d strains used. All changes occurred with predictable regularity and were inherited by the progeny. In order to compare the frequency, in R and S populations, of cells susceptible of transformation, induction of type c from type d populations was used as a tool. Two technics described previously(4) were used for this comparison: 1) Study of the influence of population size when the transformation proceeds during growth for 48 hours and 2) Examination of populations of different sizes for the presence of initially susceptible cells; the transforming principle is destroyed by desoxyribonuclease after an exposure period of 5 minutes which is adequate for transformation.

In Table I are listed the results of three experiments which show the population sizes of type specific d cells in which transformation

TABLE I. Influence of Size of Population of Type d *H. influenzae* on Induction of Type c During 48 Hr Growth Period in Levinthal Broth Plus Type d Antibody.

	Approximate population sizes		
Exp.	1.0×10^6	1.0×10^4	1.0×10^2
1	+ (2)	+ (2)	+ (2)
2	+ (3)	+ (3)	— (2)
3	+ (3)	+ (3)	+ (3)

+ = Type c present.
— = No type c found.

No. in parentheses of either + or — symbols indicates No. of samples examined in each experiment.

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* This work was supported by grants from the National Institutes of Health.

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5. Brown, J. H., *Science*, 1926, v64, 429; also personal communication to the author.

TABLE II. Influence of Population Size of Type d *H. influenzae* on Presence of Cells Initially Susceptible of Transformation to Type c.

Transforming principle	Approximate population sizes		
	1.0×10^6	1.0×10^5	1.0×10^4
TPC	— (1) + (4)	— (1) + (4)	— (5)
TPC	+ (3) — (2)	+ (3) — (2)	— (5)

TPC = Transforming principle isolated from type c.

+ = Type c present.

— = No type c found.

No. in parentheses of either + or — symbols indicates No. of samples examined in each experiment.

occurs during a 48-hour growth period. They do not differ significantly from the results obtained with R cells derived from type d under comparable circumstances.

In Table II are listed data which determine the population sizes of type d which must be used in order to ensure the initial presence of susceptible cells. Here again, no significant difference is demonstrated between R and S cell population sizes which contain susceptible cells prior to growth.

Discussion. The slightly greater degree of irregularity seen in these experiments on S cells, as compared with previously published observations on R cells, may be explained by the fact that the identification of presence of new types is much less efficient because of the large number of recipient cells; the specific antibody concentration in the transforming environment is too low to separate completely the recipient cells from the induced new type by agglutination and sedimentation. A stronger concentration can be expected to increase greatly the efficiency of detection of induced new type specific cells.

Since the type d populations used for induction of type c were derived directly from a single clone (colony), the populations can be viewed as homogeneous with respect to the type specific trait. Moreover it was very difficult to demonstrate the presence of an R colony before exposure to the transforming environment. Therefore, if susceptible cells arise by mutation, they are unlikely to be derived solely as mutants of R cells. The factor responsible for susceptibility must arise independently of the R and S genetic traits, for it has not been possible to demonstrate a significant difference in the frequency of susceptible cells in R and S populations.

Summary. 1. Normal strains of types b and d *H. influenzae* have been directly transformed to new types of *H. influenzae;* type b was changed to types a, c and d, and type d into types a, b and c. 2. The rate of occurrence in type d populations of cells susceptible of transformation has been shown to be not significantly different from that found earlier in R cell populations derived from type d. Comparisons in the presence of more potent anti d antibody have been made subsequently between populations of Rd and type d for frequency of cells susceptible (initially or during growth for 24 hours) to transformation to type c. No real differences could be demonstrated when this more efficient selective agent was used.

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Hemodilution as a Result of Estrogen Therapy. Estrogenic Effects in the Human Female. (19162)

CARROLL L. WITTEN AND JAMES T. BRADBURY.

From the Institute of Medical Research and Department of Obstetrics and Gynecology, University of Louisville, Ky.

Many articles concerning the effects of estrogenic substances on the so-called "blood picture" may be found in the literature. There are numerous publications concerning the effects of estrogens on the blood composition of laboratory animals. Vollmer and Gordon(1), Tyslowitz *et al.*(2,3), Castrodale(4), and

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Crafts(5) reported various experiments in the rat, dog, and monkey. All recorded a drop in erythrocyte count following the injection of estrogens and some reported a lowered level of hemoglobin. Friedlander, Laskey, and Silbert(6), in a study on bilaterally oophorectomized women, recorded a reduction of about 25% in blood volume from the pre-operational level. This loss in blood volume was readily restored by small doses of estrogen. Kretzschmar and Barnes(7) in the early clinical testing of diethylstilbestrol reported a slight lowering of red cell count, white cell count, and hemoglobin, and postulated that "while such a drop was within the limits of technical accuracy, it might be a reflection of the increase in blood volume which is said to occur with the administration of estrogenic substances." Karnaky(8), in a study of 65 patients being treated with stilbestrol, concluded that "the blood picture is not altered even with a dosage of 181,960 mg of stilbestrol over a period of slightly less than a year." His figures, however, reveal a lower erythrocyte count and hemoglobin in the stilbestrol-treated women. He explained this apparent difference by the statement that some of those receiving stilbestrol were menometrorrhagic cases, so a lowered red cell count and hemoglobin might be expected. He compared the average blood counts of the estrogen-treated group with those of an untreated group, but did not record the changes in any patient before and during treatment. In contrast to Karnaky's claims are those of Bateman(9) who reported a moderate anemia in 7 out of 10 women treated with stilbestrol for carcinoma. Bateman also noted that the blood volume was increased as much as 40% above the normal and that it decreased after discontinuing the stilbestrol therapy. Hamblen(10) states that "blood volume is increased due to alterations in electrolyte and water metabolism resulting from administration of estrogens," but on another page makes the contrary statement that, "although estrogens favor the storage of water and electrolytes, the water retention is not intense enough to produce hemodilution."

It was deemed of value to determine, under controlled conditions, the effects of estrogens on the blood of the human female—with the thought in mind that some correlations with the vasomotor symptoms of post-menopausal syndrome might be forthcoming. Also, with the present widespread use of estrogenic substances, it is of interest to understand some of the effects of this ovarian hormone on extragenital tissues.

Methods. This paper summarizes the observations on 16 female outpatients of the Louisville General Hospital, to whom estrogens were administered. These women were suffering from minor menstrual irregularities and varied in age from 15 to 52 years. In the younger women, the estrogen treatment and the blood studies were completed in the inter-menstrual interval so none of the blood changes could be attributed to menstrual blood loss. Venous blood samples were drawn on each visit and the red cell count, hemoglobin, and hematocrit were determined. At least 2 control determinations were obtained before estrogens were administered on alternate days for a total of 2 to 4 intramuscular injections. The estrogen dosage consisted of

TABLE I. Changes in Blood Composition During Estrogen Therapy.

	Erythrocyte count	Hemoglobin, g	Hematocrit, mm
Max decrease	1110000	1.9	11
Min "	210000	.4	2
Avg "	665000	1.1	6
Avg % decrease	14.8%	8.5%	15%

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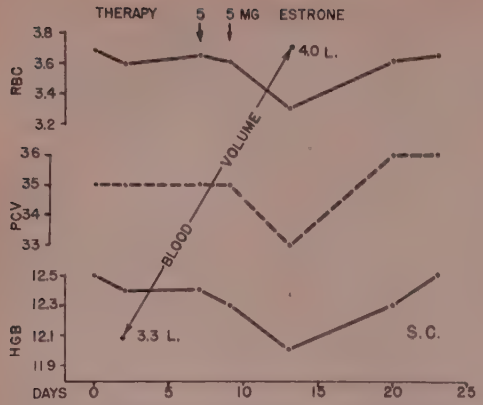

FIG. 1. Changes in blood composition and blood volume as result of 2 injections of estrone on alternate days.

either 5 mg of Theelin (Estrone) or 0.4 mg of α-estradiol dipropionate per injection. On 3 of the 16 patients, blood volumes were determined by the intravenous injection of Evans Blue T-1824 dye before and during estrogen treatment.

Red cell counts were made using standardized pipettes, Hayem's solution and a Spencer Bright Line counting chamber. *Hemoglobin* was determined as acid hematin by a standardized procedure utilizing the electrophotometer. *Hematocrit comparisons* were obtained from oxalated blood in Wintrobe tubes centrifuged for 30 minutes at 3000 r.p.m. *Blood volume* was determined by (a) preparing a standard by the addition of 0.1 cc of a 1:100 dilution of 1% T-1824 Evans Blue dye to an accurately pipetted 5 cc portion of heparinized whole blood. (b) injecting 1 cc of 1% T-1824 Evans Blue dye into the antecubital vein and 5 minutes later withdrawing 5 cc of blood from the other arm and placing the venous blood into a heparinized tube. (c) centrifuging the 2 blood specimens (a) and (b) to obtain the plasma. (d) comparing the relative concentration of Evans Blue dye in the plasma of the standard and the unknown at 620 mμ in the electrophotometer, and computing therefrom the total blood volume.

Results. In the 16 patients there occurred a consistent lowering of the erythrocyte count, hemoglobin and hematocrit during the 4 to 10 days in which estrogen was administered. On cessation of the estrogen therapy there resulted a subsequent return of initial levels within a period of 5 to 9 days. The magnitude of these changes is summarized in Table I.

The trends presented may best be illustrated graphically. The first 3 figures (Fig. 1-3) are those of 3 patients of this series and illustrate the lowering of the erythrocyte count, hemoglobin, and hematocrit correlated with the computed blood volume changes during estrogen administration. The increases in blood volumes in these 3 patients (21%, 19%, and 15%, respectively) demonstrate

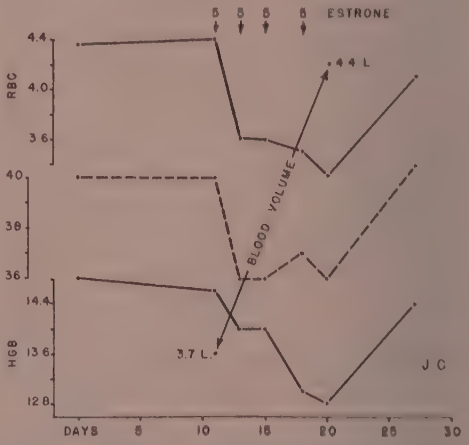

FIG. 2. Changes in blood composition and blood volume as result of 8 injections of estrone on alternate days.

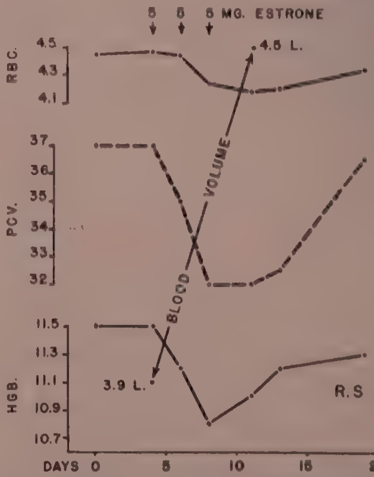

FIG. 3. Changes in blood composition and blood volume as result of 4 injections of estrone in 1 wk.

TABLE II. Total Circulating Red Cell Volumes in Three Patients Before and During Estrogen Treatment. Calculated from the hematocrits and the blood volume estimations based on injections of Evans Blue dye.

	Total circulating red cell volume	
Patient	Before estrogen, l	During estrogen, l
S. C.	1.15	1.32
J. C.	1.48	1.58
R. S.	1.44	1.49

that the decreases in red cell count, hemoglobin and hematocrit were due to a hemodilution produced by a marked increase in plasma volume. When the hematocrit values obtained on the day of blood volume determinations were used to determine the total volume of circulating red blood cells, it was obvious that there was no appreciable increase in the volume of red cells during the experiment (Table II). Similar changes in the composition of the blood were noted after another estrogen (estradiol dipropionate), Fig. 4 and 5.

Discussion. The studies confirm the earlier observations of other authors who have reported changes in blood composition which may have been due to an increased blood volume in laboratory animals treated with estrogen. The loss of blood volume following oophorectomy as noted by Friedlander *et al.*, and its correction by estrogen therapy suggests that the vasomotor symptoms of the surgical or natural menopause may be, in part, a reflection of blood volume changes. The hemodilution observed in our studies also suggests the possibility that the increased estrogen level during pregnancy may be a causative factor of the increased blood volume and the "physiologic anemia" that occurs in pregnant women. Studies in women who experience a definite premenstrual edema would be of interest to determine whether an intravascular fluid increase parallels the extravascular fluid increase. Hematological studies during therapy with estrogen and progesterone or testosterone would show whether these hormones modify or augment the action of estrogen.

Summary. (1) In all 16 patients in this study there was a lowering of the erythrocyte count, hemoglobin and hematocrit during the 4 to 10 days that estrogen was administered. On cessation of estrogen therapy there was a subsequent return to the initial blood constituent levels within a period of 5 to 8 days. (2) Evans Blue dye studies made in 3 of the 16 women demonstrated that the blood volume increased sufficiently to account for the lowering of the red cell count, hemoglobin and hematocrit. (3) During short periods of estrogen therapy there may be a significant hemodilution. Further studies are necessary to determine whether this effect is transitory or whether it may be maintained indefinitely.

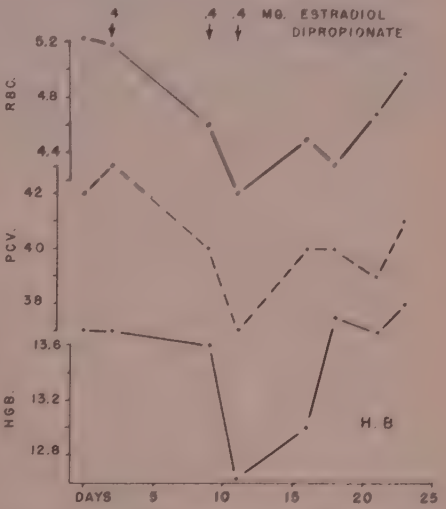

FIG. 4. Changes in blood composition as result of 3 injections of estradiol dipropionate in 8 days.

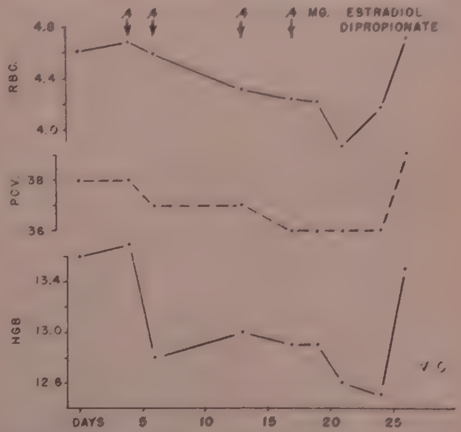

FIG. 5. Changes in blood composition as result of 4 injections of estradiol dipropionate in 10 days.

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Morphological Observations by Electron Microscopy of West Nile Virus after Propagation in the Swiss Mouse. (19163)

REGINALD L. REAGAN AND A. L. BRUECKNER.

From the Live Stock Sanitary Service Laboratory, Maryland State Board of Agriculture, University of Md., College Park.

The West Nile strain (B 956) of virus used for this study was furnished by the American Type Culture Collection of Washington, D. C. This virus had originally been obtained from Dr. K. C. Smithburn of the Rockefeller Foundation Laboratories in New York(1). He isolated this strain in 1937 from the blood of a patient in Uganda, Africa. On receipt at this laboratory the virus had been passed 25 times in Swiss Albino mice by the intracerebral route of inoculation, and at this laboratory it was passed one additional time before initiating the experiment.

Six Swiss albino mice (3-weeks-old) were inoculated intracerebrally with 0.3 cc of a 10% suspension of West Nile virus of the 25th intracerebral mouse passage. The virus received here was lyophilized mouse brain and was diluted to a 10% suspension with sterile distilled water. The mice showed nervous symptoms of involuntary motor reactions on the 4th day. When symptoms appeared the mice were sacrificed, and the brains and cords were removed aseptically. Six mice were injected intracerebrally with a 10% suspension of normal mouse brains. No symptoms were noted in these normal controls. These 6 mice were sacrificed on the 4th day post inoculation. The brains and cords were removed aseptically as above. The pools of infected mouse brains and cords and of normal mouse brains and cords were ground with alundum, and diluted to 10% suspensions with physiological saline. The 2 suspensions were then subjected to 5 minutes centrifugation in an angle centrifuge at 2000 rpm. The supernatants were removed from the specimens and filtered through type ST size L3 Seitz filters. The filtrates were then subjected to centrifugation for 2 hours at 44,770 rpm under refrigeration. The temperature of the refrigerated outer jacket stayed constant at –8°C during the centrifugation. After the 2-hour centrifugation period, the supernatants from the specimens were discarded and the sediment from each specimen was resuspended in 0.8 cc of physiological saline. Several drops of each suspension were placed on several parlodion film supports which had been prepared 24 hours previously. After all excess fluid had been removed with small capillary pipettes, the films were dried and shadowed with chromium(2) at arc tangent 2/10 and examined under the RCA electron microscope, type EMU. The balance of the material of each suspension was injected intracerebrally into each of six 3-week-old Swiss albino mice.

The mice injected with the concentrated infected material showed nervous symptoms after a period of 6 days. These mice were sacrificed and the brains were removed aseptically, pooled, ground, and diluted to a 10% suspension. This suspension was injected intracerebrally into 6 unvaccinated mice and into 6 mice which previously had been immunized intramuscularly with the original West Nile virus. After 8 days the unvaccinated mice showed symptoms of central nervous system involvement while the immunized group showed no nervous symptoms. This confirmed the concentrated virus to be West Nile virus. The mice injected intracerebrally with the concentrated suspension of normal mouse brain and cord appeared normal and were discarded after a 21-day observation period.

Upon electron microscope examination of the concentrated suspension of the brains and cords of the normal mice, no virus-like particles could be seen. These controls were screened carefully. Upon examination of the concentrated suspension from the infected

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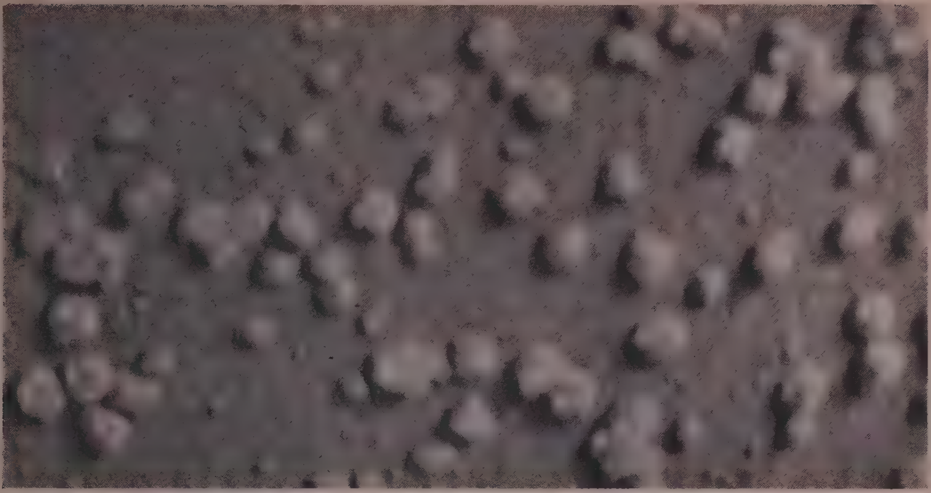

FIG. 1. West Nile Virus shadowed with chromium at arc tangent 2/10. × 150,000.

brains and cords, uniform virus-like particles were demonstrated as shown in Fig. 1. These bodies measure 38-40 mμ by direct measurement and are cube-shaped with irregular contour.

Summary. Studies by electron microscope of brains and spinal cords of mice infected with West Nile virus, B 956 strain, show the virus to be cube-shaped with a diameter of 38-40 mμ. These bodies could not be demonstrated in concentrated normal mice brains and spinal cords subjected to the same procedure of concentration and examination. The bodies from the West Nile preparations resemble the rabbit papilloma virus described by Sharp *et al.*(3). The virus in the concentrated material was infectious for unvaccinated mice but had no effect on mice previously immunized against the West Nile virus.

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Composition of Mitochondrial Preparations in Relation to Intracellular Localization of Azoprotein in the Mouse.* (19164)

DAVID GITLIN,[†] BENJAMIN H. LANDING, AND ANN WHIPPLE.
(Introduced by J. F. Enders.)

From the Departments of Pediatrics and Pathology, Harvard Medical School and The Children's Medical Center, and the Histochemistry Laboratory of the Children's Cancer Research Foundation, Boston, Mass.

The tissue localization of labeled proteins has been the subject of extensive investigation, and after parenteral administration, such antigens have been demonstrated repeatedly in the cells of the reticulo-endothelial system and, to a lesser extent, in those of other organs (1-6). It is known that the distribution of

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[†] Post-Doctorate Research Fellow, Public Health Service, National Institutes of Health.

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azoproteins in the tissues of mice and rabbits depends on both the protein and the azo-dye moieties(5,7), and that such proteins are found in the cytoplasm of the involved cells, chiefly in the form of small granules(1-3,5). Recently, Crampton and Haurowitz(8) reported the bulk of injected radio-iodinated antigen to be in the mitochondrial fraction, and suggested that mitochondria are the site of antibody synthesis. In this regard, it seemed of interest to investigate the composition of mitochondrial preparations in an effort to determine whether the small cytoplasmic granules seen after administration of azoproteins are labeled mitochondria.

Materials and methods. Groups of 3 or 4 CF_1 white mice were injected intravenously with 5 to 20 mg of one of the following azoproteins: acriflavine azo-human serum albumin (AHA); acriflavine azo-bovine serum gamma globulin (AGG); and Evans' blue azo-human serum albumin (EBA). (The preparation of these azoproteins is described elsewhere)(9). The animals were sacrificed approximately eighteen hours after intravenous injection of the labeled protein. Microscopic sections showed that the acriflavine azoproteins were localized chiefly in the Kupffer cells of the liver, and the Evans' blue azoprotein in the Kupffer cells and in the cytoplasm of the proximal convoluted tubules of the kidney. The livers of each group were pooled, chilled by being placed in saline solution at 0°C for 10 minutes, and rinsed with chilled normal saline solution and then with chilled 0.88 molar sucrose solution. All subsequent operations were performed at 0°C. The livers were homogenized in a Waring Blendor with 55 ml of 0.88 molar sucrose solution for 45 seconds. The technic for preparation of mitochondrial fractions was essentially that described by Hogeboom and his associates (10). The liver suspensions were centrifuged 3 times at 1,000 G for 15 minutes, and 15 ml aliquots of the final supernatant were centrifuged twice at 20,000 G for 20 minutes in lusteroid tubes. The precipitate in one tube of each group was fixed *in situ*, using a 10% solution of formalin in 0.88 molar sucrose; a duplicate precipitate was resuspended in 0.88 molar sucrose for color determinations. In some instances, a third tube of each lot was centrifuged 5 times at 20,000 G for 20 minutes, and the precipitate fixed *in situ*. Three sets of controls were used: pooled livers of normal mice; pooled livers of mice injected intravenously with 0.025 ml of Higgins' India Ink, and pooled livers of normal mice, to the mitochondrial fraction of which the three azoproteins were added in one-half the amount used for injection. After fixation for 24 hours in the lusteroid tubes, the precipitates were dehydrated and cleared in alcohol and xylol. During this procedure, the tubes were dissolved, leaving the precipitates as thin wafers, which were embedded in paraffin and sectioned. Some sections were examined unstained, whereas others were stained with Altmann's fuchsin, Mallory's phosphotungstic acid hematoxylin, and Regaud's iron hematoxylin methods for mitochondria(11). Sections from various wafers were also stained with McManus' periodate leucofuchsin procedure for carbinols, Gömöri's ferric and ferrous iron procedures, the Prussian blue reaction for sulfhydryl groups, and the methylgreen pyronine procedure for nucleoproteins (12-15).

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TABLE I. Results of Rough Color Comparisons of Azoprotein Content of Various Fractions.

	% of color of whole liver homogenate			
			Controls with azoprotein added to whole liver homogenate	
Fraction	Inj. azoprotein (AHA, AGG, or EBA)	India ink	Washed twice	Washed 5 times
Precipitate after suspension centrifuged 3 times at 1000 G for 15 min	10-20	50-60	5-10	5-10
Mitochondrial fraction	40-50	30-40	5-10	0
First supernatant of mitochondrial fraction	30-40	0-10	80-90	80-90

Results. The results of rough color comparisons of the azoprotein content of the various fractions, using azoprotein solution standards, are indicated in Table I. The distribution parallels that obtained by Crampton and Haurowitz with radio-iodinated proteins in rabbits(8). As can be seen, a relatively large fraction of the injected azoprotein was found in the mitochondrial fraction; however, this was also true of injected India ink. Azoproteins added to the whole liver homogenate were not found in the mitochondrial fraction in significant amounts.

Unstained sections of the wafers prepared from the mitochondrial fractions always showed the azoprotein granules to be concentrated in a narrow zone on the centrifugal edge (Fig. 1), whereas India ink particles formed 2 bands, one on the centrifugal edge and one in the center of the wafer (Fig. 2). Occasionally, the azoprotein layer was discontinuous, reflecting a stellate pattern of dye seen in the whole wafer (Fig. 1, 3 and 4). All 3 mitochondrial stains showed stratification of the material in the wafers; however, the band pattern seen was not the same when the various stains were used on sections of the same wafer, nor was it consistent from one preparation to another. Examples of this are seen in Fig. 3, 4, 5 and 6. In general, the Altmann procedure stained the most granules and the phosphotungstic acid hematoxylin procedure the fewest, whereas the iron hematoxylin procedure gave the best defined granules. In favorable preparations with the 2 hematoxylin stains, the acriflavine-protein colored granules could be seen to form a band which did not take the stain, and with the Altmann stain, Evans' blue-protein colored granules could be seen in a poorly defined zone among the red particles of the fraction. The ferric iron procedure, the only one of the histochemical methods employed which demonstrated stratification in the wafers, did not give bands agreeing with any of the three mitochondrial stains. In some preparations, the McManus procedure stained varying numbers of large irregular structures, presumably small glycogen granules.

Conclusions. These studies substantiate previous reports that a relatively large proportion of injected labeled protein is found in the mitochondrial fraction of homogenized liver(8). Failure to demonstrate in this fraction the azoprotein which was added to the whole liver homogenate indicates that an *in vivo* process is involved and that simple adsorption of the azoprotein by mitochondria does not occur. Results of microscopic study of wafers, made by centrifuging mitochondrial fractions, indicate that the intracytoplasmic granules of azoprotein seen in sections from injected animals(1-3,5) are found predominantly in such mitochondrial fractions. However, the facts that injected India ink particles occur in a similar proportion in such fractions, that the azoprotein granules do not take various mitochondrial stains, and that other impurities can be demonstrated in such preparations, indicate that such mitochondrial fractions do not consist only of mitochondria. This conclusion has also been expressed by previous workers, and such fractions have been subfractionated(16). The differences in re-

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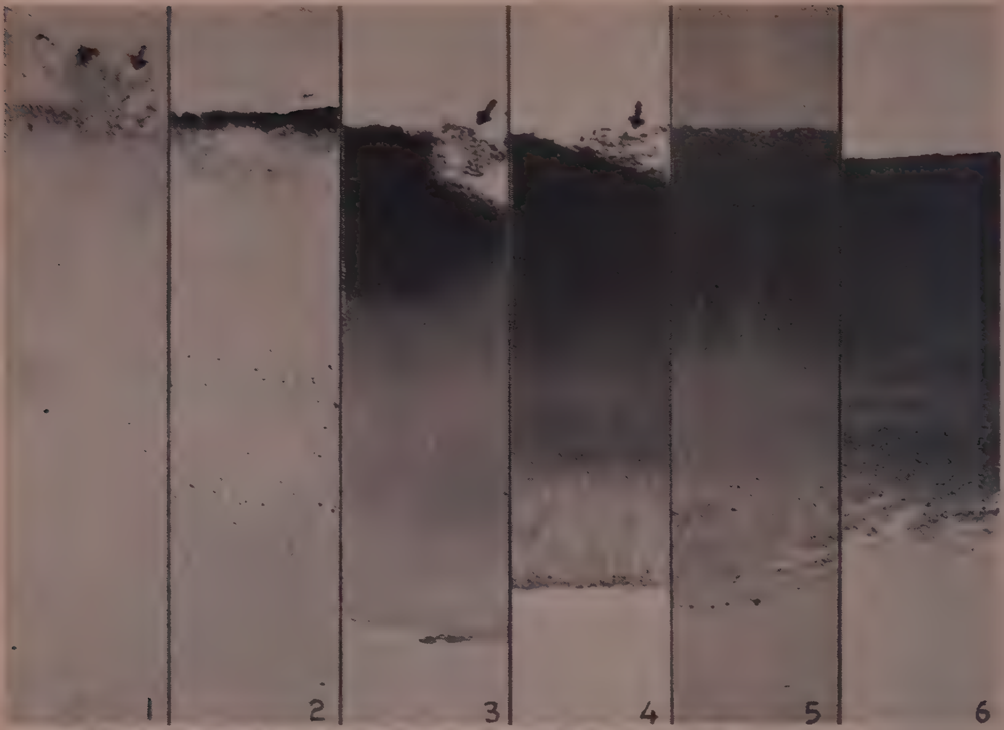

FIG. 1. Unstained section of wafer of sedimented mitochondrial fraction after intravenous injection of acriflavine azo-human serum albumin (AHA), showing concentration of labeled protein on centrifugal (upper) border (× 183). Cleft (arrow) contains loosely packed stained granules, many of which have washed out.

FIG. 2. Unstained section of wafer after injection of India ink, showing bands on centrifugal border and in center of wafer (× 183).

FIG. 3. Same wafer as in Fig. 1, stained with phosphotungstic acid hematoxylin. Note depth of stain along centrifugal border, faint stain of granules in cleft (arrow), and bands in wafer (× 183).

FIG. 4. Same wafer as in Fig. 1 and 3, stained with Altmann's fuchsin stain, showing more extensive stain than with phosphotungstic acid hematoxylin and poor definition of bands (× 183). Granules in cleft (arrow) are fainter than the stained zone.

FIG. 5. Phosphotungstic acid hematoxylin stain of wafer after injection of acriflavine azo-human serum albumin (AHA) and Evans' blue azo-human serum albumin (EBA). A narrow dark line of unstained AHA labeled granules is seen along the centrifugal border (× 183).

FIG. 6. Same wafer as in Fig. 5, stained with iron hematoxylin. Note dark staining of pale band of Fig. 5 (× 183).

sults with the various mitochondrial stains not only emphasize the inhomogeneity of these preparations, but indicate that such stains do not provide adequate characterization of mitochondria. It is apparent that much more work must be done on the composition of mitochondrial fractions prepared in this way, and on the significance of the results of mitochondrial stains, before cytological and biochemical data on such preparations can be equated with assurance. Since intracytoplasmic azoprotein granules apparently differ in some respects, both physically and histochemically, from other granules in these preparations which fulfill more of the criteria for mitochondria, the occurrence of labeled proteins in mitochondrial fractions does not as yet seem to justify the conclusion that these proteins are attached to mitochondria, or that mitochondria are the site of antibody formation.

Summary. The results of colorimetric and microscopic studies on cell fractions prepared after intravenous administration of various azoproteins to mice are presented. The azoprotein granules seen intracellularly in the tissues of such animals are present predominantly in the mitochondrial fraction. Evidence that such granules are not labeled mitochondria is presented, and the significance of the variation in the results of different staining technics on mitochondrial fractions is discussed with regard to the homogeneity of these preparations.

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A Semi-Synthetic Medium for Labelling Hemolytic Streptococci with Radioactive Phosphorus.* (19165)

SUNG J. LIAO AND W. A. KREHL.

From the Section of Preventive Medicine and the Yale Nutrition Laboratory, Department of Physiological Chemistry, Yale University School of Medicine, New Haven, Conn.

Although several "synthetic" media for the cultivation of group A hemolytic streptococci, such as those of Bernheimer *et al.*(1), Christensen(2), Stock(3), Dole(4), and Slade and Knox(5), have been reported and appear to be satisfactory for the growth of these bacteria, they are not suitable for studies which require the incorporation of radioactive phosphorus in the bacterial cell. This is due to the fact that their buffer and amino acid nitrogen sources contribute an excessive amount of ordinary phosphorus which would compete with the radioactive form of the element in metabolic processes of the organism.

It is the purpose of the present paper to report the development of a medium which will support excellent growth of streptococci in the presence of a small, but optimal amount of phosphorus in the form of radioactive phosphoric acid.†

Materials and methods. Organisms. The present study was done mainly with group A hemolytic streptococci type 18 which was isolated from the throat of a patient with active rheumatic fever. In the later parts of this study, group A hemolytic streptococcus type 14, which is a mouse-virulent strain (S23/101/4),‡ was also used. The purity of these strains was checked by serological typing at various times during the progress of the experiments. *Inoculum.* A platinum loop, 3 mm in diameter, was used throughout the study. A loopful of an overnight culture of the streptococcus was inoculated into 10 ml of the semi-synthetic medium. The organisms were maintained at first in a serum broth culture. When equivalent growth was finally obtained with the semi-synthetic medium, it was always substituted so as to avoid any possible transfer of traces of substances in the serum broth which might stimulate the bacterial growth. *Criteria of growth.* The appearance of turbidity to the naked eye after cultivation at 37°C for 15 to 18 hours, and the presence of only Gram positive cocci in chains on smear were taken as evidences of growth. Simultaneously with each set of experiments with semi-synthetic media, a tube of serum broth was seeded to serve as a control for the viability of the organisms in the inoculum. *Sterilization of the medium.* After the solid constituents were dissolved, the pH of the solution was adjusted to 7.8; the final volume was made up with distilled water and the

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‡ Kindly given by Dr. Rebecca Lancefield

TABLE I. Composition of the Proposed Medium.[*]

Enzyme hydrolyzed lactalbumin[†]	2 g[‡]
Glucose	250 mg
DL-tryptophane	10
L-cystine	10
Glutamine	5
Adenine sulphate $2H_2O$	1
Guanine·HCl·$2H_2O$	1
Uracil	1
Xanthine	1
Ascorbic acid	20
Biotin	5 μg
Folic acid	5
PABA	50
Riboflavin	100
Thiamin	100
Pyridoxine	100
Nicotinic acid	100
Ca pantothenate	100
$MgSO_4 \cdot 7H_2O$	20 mg
NaCl	4
$FeSO_4 \cdot 7H_2O$	4
$MnSO_4 \cdot H_2O$	12
Na acetate	200
KCl	50
$Na_2CO_3 \cdot H_2O$	200
K_2HPC_4	as required[†]
Distilled water *ad*	100 ml

[*] Tryptophane and cystine were first dissolved in several ml of water with the aid of a few drops of conc. HCl. Before addition of sodium carbonate to the rest of the mixture, pH of solution should be adjusted between 5 and 6 to prevent decomposition of carbonate. After pH of complete medium was adjusted to 7.8, water was added to the required volume.

[†] See text.

[‡] The hydrolysate prepared (see text) contained about 80 mg of solids per ml.

entire medium was sterilized by filtration through a Seitz EK pad. Appropriate steps were taken to insure the sterility of the medium before it was used in experiments. *Determination of phosphorus.* Samples were digested with a mixture of equal volumes of conc. H_2SO_4 and conc. HNO_3 (analytical reagents) on a hot plate until the fluid became watery clear and all the residual NO_2 was expelled. The Fiske-Subbarow method (6) was utilized to determine the amount of phosphorus in the sample.

Experimental. Constituents of the proposed medium. Although many of the constituents of the medium used in this study are similar in kind and amount to those of other workers as referred to above, (the complete composition of the proposed medium is listed in Table I), important modifications in the nitrogen and buffer sources and minor alterations in the rest of the medium have been made. It should be noted, for example, that the present medium incorporates the same amounts of purines, pyrimidines, vitamins, and minerals which have been used with uniform success for the cultivation of lactobacilli(7). Sodium acetate was used because of its potential value as a bacterial growth-stimulant(8,9). Glutamine was included to enhance the rapid growth of streptococcus. Glutamic acid failed to replace glutamine for this purpose, as has been observed previously(10). The amino acids, tryptophane and cystine, were included to avoid their possible loss during the preparation of the protein hydrolysate. Ascorbic acid served equally well as thioglycollic acid for the maintenance of the proper O/R conditions.

A buffer other than phosphates. Two possible buffers, other than phosphate, effective at pH 7.6 or 7.8 are sodium diethyl barbiturate and borate. Sodium diethyl barbiturate was tried in amounts varying from 16 to 75 mg per 100 ml medium, but no growth was observed after 48 hours' cultivation at 37°C. Because borate is potentially toxic in the quantities needed for adequate buffering action at the pH range required, no trial was made to establish its practical value as a buffer in the present experiments. Finally, sodium carbonate was used at a level of 200 mg ($Na_2CO \cdot H_2O$) per 100 ml medium. Excellent growth of the streptococcus was obtained, and the final pH of the culture after 24 hours incubation at 37°C was 6.5. For sake of economy in this series of experiments, the organic nitrogen source was enzyme hydrolyzed casein (2 g per 100 ml medium), and phosphorus was added as K_2HPO_4 in the amount of 50 mg per 100 ml medium.

Lactalbumin hydrolysate as organic nitrogen source. 1) Removal of phosphorus. Although pure lactalbumin contains no phos-

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phorus, this element was found to be present as an impurity in the ordinary commercial samples of lactalbumin. The removal of this phosphorus was essential in order to obtain a "phosphorus-free" preparation. Two hundred g of lactalbumin were washed by stirring vigorously for 30 minutes with 5 volumes of 50% ethanol, followed by filtration through a Buechner funnel. After 3 such washings and filtrations, the material was washed once with 95% ethanol, filtered, and dried in a hot air oven at 90°C-100°C overnight. No detectable amounts of phosphorus could be found in 1 and 5 g samples of the refined preparations.

(2) *Preparation of enzyme hydrolysate.* To insure adequate enzymatic hydrolysis of lactalbumin, both peptic and pancreatic hydrolysis was applied. One hundred g of lactalbumin (phosphorus-free) was suspended in 1 liter of distilled water and the pH was adjusted with HCl to between 1 and 1.5. One gram of pepsin (U.S.P. reference sample) was added. The suspension was covered with a thin layer of toluene and incubated for 48-60 hr at 37°C. At the end of this period, the pH of the digest was found to be 2.5. The peptic digestion was terminated by adjustment of the pH of the preparation to 6 with NaOH, 8 g of $NaHCO_8$ was then added, and the pH further adjusted to 8. One gram of pancreatin (U.S.P.) was added and hydrolysis continued for another 48-60 hr at 37°C. No significant change in pH of the hydrolysate was observed during this period. Since certain batches of hydrolysate, which were steamed and clarified with activated carbon according to the method of Roberts and Snell(11), gave inconsistent results, the latter step of clearing with activated carbon was omitted from the present procedure. At the end of the pancreatic digestion, the pH of the fluid was brought to 3.5 with HCl and steamed for 15 minutes. Precipitates were removed by centrifugation and filtration. The filtrate was clear and light brown in color and contained about 80 mg of solids per ml. Batches of lactalbumin hydrolysate so prepared have not failed as a nitrogen source for good growth of the organism. With 100 ml of the proposed medium (Table I), containing 40 ml of this hydrolysate and 5 mg K_2HPO_4, a 15-hr culture contained about 650 million streptococci per ml (determined turbidimetrically against Brown's $BaSO_4$ standards)(12). When K_2HPO_4 was excluded from this medium, no detectable amounts of P could be found in the preparation and no growth of streptococcus was observed.

Trials of hydrolysis of lactalbumin with pancreatin alone were made. Such a product contained only about 16 mg of solids per ml and failed to support the growth of the organism. Both amino acids and acid hydrolyzed proteins (egg albumin and casein) as sources of amino nitrogen were tried but without success. When, however, liver fraction "L" was added at a level of 10 mg per tube, good growth was obtained with either amino acids or acid hydrolyzed proteins. This may serve as an evidence to emphasize the importance of a peptide-like substance, strepoginin, for the metabolism of group A hemolytic streptococci(13).

Amounts of potassium and phosphorus required for growth. It was evident that the amount of potassium became limited as K_2HPO_4 was eliminated from the medium. The use of 5 mg each of KCl and K_2HPO_4 per 100 ml of medium did not result in a maximum growth. When the level of KCl was increased to 50 mg per 100 ml medium, excellent growth was again noted. No exact determination was made as to the optimal amount of K required by the organisms for maximum growth. With the medium described in Table I, it was found in a series of experiments that as little as 3.0 mg of K_2HPO_4 (equivalent to 55.2 μg P) per 100 ml medium resulted in maximum growth of the streptococcus while with 0.5 mg K_2HPO_4 (equivalent to 9.2 μg P) per 100 ml medium, suboptimal but definite growth was observed.

Tagging of streptococcus with P32. Attempts were then made to label streptococcus

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with radiophosphorus. Ten ml of the proposed medium (Table I) containing 0.3 mg K_2HPO_4 was used for each experiment. One hundred μc of carrier-free P32 was added to the medium. To ensure better utilization of phosphorus, 1 ml of an 18-hr culture of type 14 hemolytic streptococcus was employed as the inoculum. Cultivation was carried out at 37°C for 4 hours. The culture was heated in a boiling waterbath for 5 minutes and centrifuged. The sediments were washed 3 times with liberal amounts of distilled water and dried in a hot-air oven at 90°C overnight. Two ml of conc. HNO_3 was used to digest the dried bacteria. The excess acid was removed by heating to dryness. The solids were dissolved with 1 ml of 1:3 HCl and 0.1 ml amounts of the solution were put on small filter paper discs (2 cm in diameter). Counting was done after the paper discs were dried. It was found that only 2% of the radioactivity added was taken up by the organism. The experiment was repeated twice with 200 and 400 μc respectively. Cultivation was prolonged to 18 hr. With these 2 trials, about 20% of the radioactivity could be recovered in the organisms. It seemed therefore that a highly radioactive streptococcus could be obtained by using a larger amount of P32 and cultivating for a longer time.

Discussion. It is evident that the proposed medium was able to support excellent growth under circumstances of a controlled supply of phosphorus. It should be of value in bacteriological and immunological studies involving the use of radiophosphorus. It is also plausible that such a medium may be used to study phosphorus metabolism of other micro-organisms.

Summary. (1) A detailed procedure for preparing a "phosphorus-free" protein hydrolysate capable of supporting excellent growth of group A hemolytic streptococci in combination with other chemically defined constituents has been described. (2) This medium has been used successfully for tagging group A hemolytic streptococci with radiophosphorus.

The writer wishes to express his gratitude to Mrs. Rebecca C. Lancefield who generously supplied strains of group A hemolytic streptococci. Thanks must also be due to Prof. John R. Paul for his interest and encouragement in this investigation.

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Lymphocytic Response of Normal Individuals to the Insulin Glucose Tolerance Test. (19166)

BRUNO W. VOLK AND SYDNEY S. LAZARUS. (Introduced by Co Tui.)

From the Division of Laboratories and Department of Medicine, Jewish Sanitarium and Hospital for Chronic Diseases, Brooklyn, N. Y.

Somogyi(1) has reported that glucose administered orally to normal persons 30 to 60 minutes after insulin results in the development of greater hyperglycemia than the administration of the same amount of glucose without insulin. This has been interpreted by Engel and Scott(2) to signify that "glucose magnified the normal homeostatic response to hypoglycemia." As this response is presumably mediated by the hormones of the anterior pituitary, the adrenal medulla and the adrenal cortex, Engel and Scott introduced the insulin glucose tolerance test for differentiating the normal from the hypopituitary or the hypoadrenal individual. Previous observations have shown that in the normal person either oral(3,4) or intravenous(5) glu-

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cose administration is accompanied by a reduction of the absolute lymphocyte count and on the other hand, that the intravenous injection of small doses of crystalline insulin evokes a rise of the absolute lymphocyte count(5). These changes in the blood lymphocyte level are also thought to be mediated by the hormones of the anterior pituitary, the adrenal medulla and the adrenal cortex. In studies on diabetic patients it was observed that the mean percentage increase of the absolute lymphocyte count above the fasting value declined with increasing degrees of impairment of the insulin tolerance test(6). These findings imply that the activity of the insulin antagonists are responsible for the increase of the absolute lymphocyte count after insulin administration and that the degree of activation of these mechanisms is reflected in the extent of the rise of the absolute lymphocyte count. Therefore, theoretically, if glucose magnifies the normal homeostatic response to hypoglycemia a greater lymphocytosis should be observed when glucose is administered after insulin than when insulin is administered alone. The present study is an attempt to determine whether this actually occurs.

Material and methods. Twelve non-diabetic individuals of average height and nutritional status received intravenously 0.075 unit of crystalline insulin per kilo of body weight. Capillary blood samples were withdrawn immediately before and at 20, 30, 45, 60, 90, 120 and 150 minutes after the insulin administration, for glucose determination, for total white blood count and for smears for differential counts. Nine individuals of this group and 5 additional persons of similar status received 0.075 unit of crystalline insulin per kilo of body weight intravenously followed 30 minutes later by 0.8 g of glucose per kilo of body weight orally. Immediately before the administration of insulin, and at 20, 30, 45, 60, 90, 120 and 150 minutes, capillary blood samples were taken for glucose determination, for total white cell count and for smears for differential counts. The blood sugar was determined by the Nelson modification of the Folin micro-method(7). The percentage of lymphocytes was established from the average of three differential counts of 300 cells each. The absolute lymphocyte count was determined by multiplying the percentage of lymphocytes by the total white cell count.

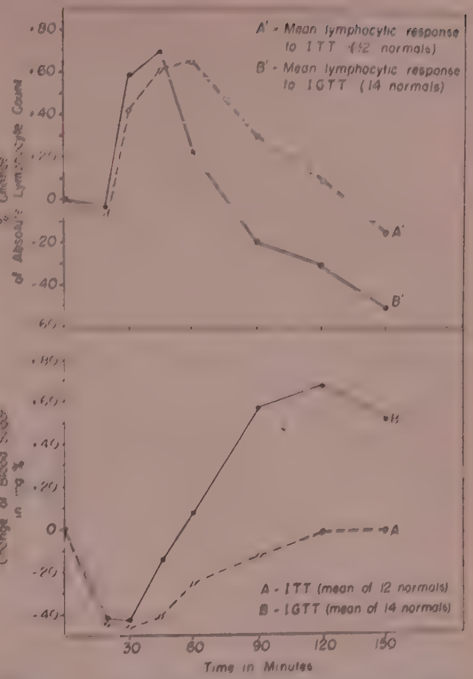

FIG. 1. Mean percentage change of absolute lymphocyte count of 12 normal persons during the insulin tolerance test and of 14 normal individuals during the insulin glucose tolerance test.

Results. Insulin tolerance test. After insulin administration, all individuals showed a decline of the blood sugar level of 40 to 60 mg %, with the nadir at 20 or 30 minutes, and a subsequent gradual rise to fasting levels at 120 or 150 minutes. All persons showed a rise of the absolute lymphocyte count of from 33% to 185% with the peak at 45 or 60 minutes except for one individual in whom the

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peak was delayed until 90 minutes. The lymphocyte values then declined gradually to or slightly below fasting level at 150 minutes. The results in these 12 normal persons are similar to those previously reported(5). The mean values for the insulin tolerance test and the corresponding absolute lymphocyte response are seen in Fig. 1 (Curve A and A^1).

Insulin glucose tolerance test. All individuals showed a decline of the blood sugar level similar to that observed in the insulin tolerance test varying from 47 to 61 mg %, the minimum value being reached at 20 or 30 minutes. It then rose to a maximum of 26 mg % to 79 mg % above the fasting level, at 90 or 120 minutes, and in one case at 150 minutes. The lymphocyte value rose in all individuals to a maximum of +41% to +178% of the fasting value at 30 or 45 minutes, and then declined to a minimum ranging from —27% to —68% of the initial value at 150 minutes. The mean values of the blood sugar and absolute lymphocyte response to the insulin glucose tolerance test are recorded graphically in Fig. 1 (Curve B and B^1).

Discussion. As can be seen from Fig. 1, the extent of the rise of the absolute lymphocyte count after insulin followed by glucose is similar to the rise observed after the injection of insulin alone. It is evident, furthermore, that there also is a conspicuous secondary decline of the lymphocyte level (Fig. 1, Curve B^1), while after insulin alone the secondary decline below the fasting level is only minimal. This difference between the two lymphocyte curves represents the effect of adding glucose at 30 minutes after insulin and the magnitude of this difference approaches that of the decline of the absolute lymphocyte value observed when the same quantity of glucose is administered by itself intravenously to normal individuals(5). The lymphocyte response to the insulin glucose tolerance test therefore represents a summation of the effects of the successive administration of insulin and of glucose, and is probably a result of stimulation of the insulin antagonists by a falling blood sugar level followed by pituitary-adrenal activation resulting from hyperglycemia. The evidence obtained from the lymphocyte response therefore does not support the hypothesis that glucose administered after insulin magnifies the homeostatic response to hypoglycemia(2). This is supported by the fact that although the rise of the blood sugar level in the insulin glucose tolerance test above the lowest value is greater than that which would be obtained in the ordinary glucose tolerance test, the maximal rise above fasting value is of the same magnitude as that observed in the glucose tolerance test. Furthermore, this is in accordance with the theoretical consideration that glucose after insulin should if anything inhibit rather than enhance the insulin antagonistic mechanisms elicited by a falling blood sugar level.

Summary. Intravenous administration of 0.075 unit of crystalline insulin per k body weight to normal individuals evoked the expected changes in the blood sugar level and was accompanied by a significant lymphocytosis. Intravenous injection of the same amount of insulin followed 30 minutes later by oral administration of 0.8 g of glucose per k body weight produced an initial decline of the blood sugar level followed by a rise above the fasting level of from 26 to 79 mg %. This was accompanied by a rise of the absolute lymphocyte count to a maximum at 30 or 45 minutes and a secondary decline to a minimum at 150 minutes. The magnitude of the difference in the lymphocyte responses to the Insulin Tolerance Test and to the Insulin Glucose Tolerance Test approaches that of the decline of the absolute lymphocyte count observed when glucose is administered alone. The change of the absolute lymphocyte count therefore does not support the thesis that glucose after insulin magnifies the homeostatic response to hypoglycemia but suggests rather that there is a summation of effects.

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Intestinal Lymphatic Transport of Absorbed Cholesterol.* (19167)

Max W. Biggs,† Meyer Friedman, and Sanford O. Byers.

From the Donner Laboratory of Medical Physics, Radiation Laboratory, University of California, Berkeley, and the Harold Brunn Institute, Mount Zion Hospital, San Francisco.

One of the many facets of cholesterol metabolism which needs clarification is more detailed information concerning the absorption and transport of ingested cholesterol. A large body of evidence has accumulated to demonstrate that oral cholesterol is partially absorbed. The quantitative aspects of this absorption, however, seem to depend upon such factors as: (1) the experimental animal used; (2) the presence or absence of fat in the diet; (3) the flow of bile; (4) the physical state of the cholesterol administered; and (5) the method of assaying the absorbed cholesterol(1-4).

Bloom and co-workers(5) have demonstrated the importance of the lymph circulation in the transport of absorbed fatty acids. Since fat and cholesterol absorption appear to be linked in some as yet undefined way, it is of importance to know whether or not the transport of absorbed cholesterol occurs via the lymphatics and thoracic duct as well. The investigation to be described herein was designed to determine whether the thoracic duct lymph is an important vehicle for the transport of absorbed cholesterol to the systemic circulation.

Experimental. The intestinal lymph was collected in normal male adult rats (Long Evans) according to the technic described by Bloom *et al.*(5). Briefly, this consisted of the introduction, under ether anesthesia, of a plastic cannula (O.D.: 0.06") into the abdominal segment of the thoracic duct. A second cannula was fixed in the stomach by a purse string suture. This stomach tube allowed the introduction of the experimental mixtures of fat and tritium-cholesterol. The animals were maintained in restraining cages. In this way, a continuous collection of lymph could be obtained from unanesthetized animals for a period of 48 hours. The rats were allowed to drink 0.4% NaCl solution *ad lib.*

Six rats were selected for the experiment. Rats No. 1 and No. 2, following cannulation of the thoracic duct, were each given 15 mg of tritium labeled cholesterol(6) (SA = 0.61 μc/mg) dissolved in 3 cc of oleic acid through the gastric cannula. The lymph flow from these 2 animals was collected over a 12 hour period. At the end of this time the animals were sacrificed following removal of a blood sample of approximately 8 cc. The lymph collected was subdivided into samples corresponding to time increments of 0 to 2 hours, 2 to 6 hours, and 6 to 12 hours. Rat No. 3 was used as a control animal. A sham operation was performed but the thoracic duct was not intubated. Fifteen mg of cholesterol (SA = 0.61 μc/mg) in 3 cc of oleic acid were administered as above. This animal was killed after 12 hours; a sample of whole blood was obtained. The samples of lymph and whole blood were processed for tritium-cholesterol specific activity as previously described(4,7). Rats No. 4 and No. 5 received 20 mg of tritium-cholesterol (SA = 0.61 μc/mg) in 4 cc of olive oil after cannulation of the thoracic duct. The lymph was collected for 48 hours in two time increments, 0 to 24 hours and 24 to 48 hours. At the end of the 48 hour experimental period the animals were sacrificed. A sample of whole blood and the livers were retained for exami-

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† Fellow, Arthritis and Rheumatism Foundation.

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TABLE I.

Rat No.	Wt, g	Sample designation	Cholesterol content	Cholesterol specific activity (μc/mg) $\times 10^{-2}$	μc in total sample cholesterol	% of administered activity in total sample cholesterol
1 2	367 307	Combined (0-2 hr) lymph	.83 mg	2.84	2.36×10^{-2}	.26
1 2		Combined (2-6 hr) lymph	2.48 "	5.38	.133	.73
1 2		Combined (6-12 hr) lymph	3.25 "	6.09	.198	1.09
1		Whole blood	73 mg %	N.D.†		
2		" "	67 "	N.D.		
3‡	301	" "	81 "	.50	approx. .113*	approx. 1.24
4	265	(0-24 hr) lymph	9.5 mg	5.98	.569	4.66
		(24-48 hr) "	5.3 "	3.46	.184	1.51
		Whole blood	80 mg %	.067	.013*	.11
		Liver	17.5 mg	.076	.013	.11
5	289	(0-24 hr) lymph	5.3 "	4.61	.246	2.02
		(24-48 hr) "	3.7 "	3.29	.121	.99
		Whole blood	73 mg %	N.D.		
		Liver	14.2 mg	.018	.003	.03
6‡	283	Whole blood	66 mg %	1.52	.322*	2.64
		Liver	19.1 mg	1.40	.267	2.19

* Estimated using a total blood volume of 7.5 cc/100 g rat.

† Non-detectable. Measured electrometer drift was less than two times background. Samples contained less than 2×10^{-4} μc/mg.

‡ Control.

nation. Rat No. 6 received only the sham operation. It was given 20 mg of tritium-cholesterol as above, and was killed at the end of 48 hours. The liver and a whole blood sample were preserved for cholesterol specific activity determination.

Results. A summary of the collected data is given in Table I. A total of 2.08% of the administered dose of tritium-cholesterol was recovered in the cholesterol of the intestinal lymph (collected for 12 hours) of rats No. 1 and No. 2; however, no tritium-cholesterol could be demonstrated in the blood of either animal. On the other hand, the blood cholesterol of the control rat (No. 3) at the end of 12 hours had a specific activity of 0.50×10^{-2} μc/mg. This activity would account for approximately 1.24% of the administered dose in the circulating blood.

The lymph cholesterol collected during the first 24 hours as well as that collected during the second 24 hours in rats No. 4 and No. 5 showed a high specific activity. The combined lymph collected during the 48 hour period contained 6.17% of the administered tritium-cholesterol in the case of rat No. 4, and 3.01% in the case of rat No. 5. The liver and whole blood cholesterol of these 2 animals contained only insignificant amounts of activity. Control animal No. 6 had tracer cholesterol in the blood and liver after 48 hours to account for approximately 4.83% of the administered dose.

The specific activities of the free and esterified components of the lymph cholesterol (first 24 hours) was determined for rats No. 4 and No. 5. (Rat No. 4: Free cholesterol—SA $= 5.01 \times 10^{-2}$ μc/mg; esterified cholesterol—SA $= 6.80 \times 10^{-2}$ μc/mg. Rat No. 5: Free cholesterol—SA $= 3.74 \times 10^{-2}$ μc/mg; esterified cholesterol—SA $= 5.56 \times 10^{-2}$ μc/mg). The esterified cholesterol contains a significantly higher specific activity than the free cholesterol.

Discussion. In the experimental animals with thoracic duct fistulae the cholesterol recovered from the lymph had a specific activity more than 45 times as great as the specific activity of the serum or liver cholesterol. The intestinal lymph must therefore be quantitatively the most important pathway for the transport of absorbed cholesterol to the serum, and hence to the body organs. Since it has been demonstrated that the blood and liver contain a large portion of the cholesterol absorbed following a labeled cholesterol meal (4), the overall data may be evaluated to show that at least 90% of the cholesterol absorbed as such during the experimental period studied was recovered in the lymph samples collected from rats No. 1, No. 2, No. 4, and No. 5. In view of the probable presence of anastamotic lymph channels draining lymph into the systemic circulation via routes which would not be shunted by a cannula in the thoracic duct, the data strongly suggest that all food cholesterol may be transported normally via the lymphatics to the systemic circulation.

Only a small fraction of the amount of cholesterol orally administered is absorbed in the intact animal and it has been demonstrated as well that cholesterol absorption occurs slowly over a period of many hours, even days(4,7). Thus, the low percentage of the administered tritium-cholesterol dose which was recovered in the lymph samples, bloods, and livers of the experimental animals was to be expected.

It is noteworthy that the highest specific activity of cholesterol recovered from the lymph, 5.98 x 10^{-2} μc/mg in the case of rat No. 4, is only approximately one-tenth the specific activity of the cholesterol fed. This large dilution must arise primarily from cholesterol of endogenous origin.

Summary. Exogenous cholesterol after its intestinal absorption was found to be conveyed into the systemic circulation by the lymph of the thoracic duct. Little or no transport occurred via the portal venous system.

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Effect of Primaquine on Experimental *Trypanosoma cruzi* Infection.* (19168)

TULIO PIZZI.† (Introduced by W. H. Taliaferro.)

From the Department of Bacteriology and Parasitology, University of Chicago.

Since 8-(isopropylaminoamylamino)-6 methoxyquinoline, pentaquine or SN 13276, has some activity against *Trypanosoma cruzi* infections in mice(1,2), it seemed desirable to test the chemically related and less toxic 8-(4-amino-1-methylbutylamino)-6 methoxyquinoline, primaquine or SN 13272, against this same trypanosome. This drug has been recently tested against vivax malaria(3). Preliminary successful results in the early stages of *T. cruzi* infection in mice are presented in this paper.

Materials and methods. A highly virulent strain of *T. cruzi* (Tulahuén strain), isolated in 1945 from a human case in the northern part of Chile, was used. Following observations on the genetic constitution of mice in relation to their susceptibility to *T. cruzi* (4), the trypanosomes have been transferred regularly every 10 days for the past year into male mice (about 8 weeks old, weighing 18-21 g) of the genetically pure C3H strain (Jackson Memorial Laboratories). After a standardized intraperitoneal injection of about 200,000 trypanosomes in 0.4 ml citrated mouse blood, trypanosomes appear in the blood in 6 to 8 hours, remain scarce for several days, suddenly increase in number at 4 to 6

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† Permanent address: Department of Parasitology, University of Chile, School of Medicine, Santiago, Chile.

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days when "slender forms" are numerous, steadily increase thereafter, reach over 1000 per mm^3 on the 9th day and kill the mice in 10 to 11 days. They invade the tissues as early as 12 hours and show a predilection for connective tissue.

Trypanosomes were counted daily by a reliable method used by the author and coworkers(4-6) which involves the use of a specially marked hemoglobin pipette. The drug was dissolved in distilled water at the rate of 1 mg ml water and was given daily *per os* at the rate of 0.25 mg.

Results. Three groups of mice were treated. To the first group (10 animals), 10 daily doses were begun on the second day; to the 2nd group (10 animals), 8 daily doses were begun on the 4th day, and to the third group (2 animals which received about 380,000 trypanosomes initially), 10 daily doses were begun on the 6th day. Each group was accompanied by a similar untreated control group of the same number. Counts of trypanosomes were made daily. Results reported here cover a period of 35 days from groups 2 and 3 and for 55 days from group 1. Whereas controls died within the expected 10 or 11 days, all treated animals in the 1st group became negative in 24 hours, and the majority remained negative. Occasional trypanosomes were usually seen in one or 2 mice for 17 days and in 7 and 6 mice, respectively, on the 11th and 12th day when the controls were dead or dying. After 17 days, occasional trypanosomes were seen in 4 to 8 mice. Treated mice in the second group also lived and showed a marked decrease in trypanosomes after 24 hr (from an average of 34 mm^3 to 7/mm^3). Thereafter, occasional trypanosomes were usually seen in 2 to 4 of the mice (average = 0.2 mm^3). The two treated mice in the third group behaved as did the mice in group 2 in spite of the fact that they contained 1568 and 1960 trypanosomes/mm^3 blood, respectively, at the time of the first treatment. Four mice in group 1 died between the 37th and 54th day, and some in groups 2 and 3 were killed on the 35th day. All had only a few parasites in their blood or tissues, but some showed a mild myocarditis.

Discussion. So far, no curative drug has been found against *T. cruzi* infections. Some few substances, *i.e.,* Bayer 7602 (Ac)(7), phenanthridine compounds(8,9) and certain aminoquinolines, particularly pentaquine(1-2) act in a suppressive manner in that they prevent the death of animals during the acute stage and decrease the number of parasites in the blood. Failure to cure the infection seems to be due to an ineffective action on the tissue stages of the parasite or to a failure to reach an effective concentration in the tissues in which the parasites are located. The results in the present paper are more critical than some previously published results because they use parasitemia in the mice instead of mortality rate of the mice(10) as a criterion and deal with a highly virulent strain of trypanosomes in a genetically pure strain of mice. They indicate that, as compared to other drugs, primaquine is more effective suppressively on early stages.

Conclusions. Primaquine showed a strong suppressive action during the early stages of experimental *T. cruzi* infection in mice. Most treated animals survived infection with a virulent strain and all showed a disappearance or marked decrease in the number of parasites present in the blood. The activity of the drug seems to be stronger than other drugs so far known to be effective against *T. cruzi* infections.

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Distribution and Excretion of Electrolytes after Acute Whole-body Irradiation Injury. I. Studies with Radiopotassium.* (19169)

JOHN Z. BOWERS† AND KENNETH G. SCOTT. (Introduced by Louis S. Goodman.)

From the Crocker Radiation Laboratory and the Division of Radiology, University of California School of Medicine, Berkeley and San Francisco, Calif.

There has been considerable speculation as to the alterations in body water and electrolytes including potassium that might occur with acute whole-body irradiation injury. There is no conclusive evidence as to the cause of early death in irradiated animals, but there is suggestive evidence that alterations in tissue and blood electrolytes may be important in this regard. Edelmann(1) administered 500, 800 and 1000 roentgens to rats, and noted at all levels an increased urinary excretion of potassium, sodium and chloride on the first and fifth days after irradiation. Failure to find any consistent trends in electrolyte balance, including serum potassium levels following an LD50 exposure in dogs, has recently been reported by Soberman and coworkers(2). However, their report was based on serial analyses of plasma electrolytes without any of the excretory or tissue determinations that should be included in a true "balance" study. Moon has reported a rise in blood potassium levels during radiation sickness(3).

In considering electrolyte alterations in irradiated animals, it is important to realize the differences in sensitivity to irradiation among the various tissues and organs of the human and animal body. Radiosensitivity is probably related directly to the rate at which cells grow and divide. Thus lymphatic tissue, blood, blood-forming organs including the spleen, germinal epithelium and the mucosa of the gastrointestinal tract are considered to be most sensitive to irradiation; liver, kidney, lung and adrenal occupy an intermediate position; smooth and striated muscle, cardiac muscle, brain, nerve and bone are considered to be resistant to irradiation injury(4). Animals dying from irradiation injury may show extensive histopathological changes in sensitive tissues, while other tissues exhibit little, if any, alteration. Our experiments were designated to follow the movement of potassium in irradiated animals.

Methods. Slonaker male rats, weighing approximately 250 g, were fed a synthetic diet which is complete for the rat. Radiopotassium prepared by cyclotron bombardment of argon with alpha particles was kindly supplied by Dr. Joseph Hamilton. Owing to the relatively short half-life of the radioisotopes K^{42} and K^{43}, which are obtained by this procedure, it was necessary to administer tracer doses which alone delivered appreciable irradiation to the control as well as the experimental animals. The animals were injected subcutaneously with the carrier-free radioactive isotopes 24 hours before sacrifice, as it is generally agreed that satisfactory equilibration is obtained in this period. There were 6 groups of 6 rats each—3 control groups and 3 experimental groups. The 3 groups of control animals received a calculated dose of 50, 85 and 175 r.e.p., respectively. The 3 experimental groups of experimental animals were given 830, 819 and 817 roentgens, respectively, by means of the X-ray machine at 215 k.v. and 15 m.a., with 1.2 mm Al and 0.5 mm Cu filters at a rate of 78 r per minute. When the irradiation from X-rays and from K^{42} and K^{43} are added, these groups of animals received a total irradiation dosage of 880 r, 904 r, and 992 r; the LD50 for these animals was approximately 800 r. The irradiation dosage provided by the X-ray machine was verified in each instance with a paraffin phantom and victoreen r meter; control animals were sham irradiated under the

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† University of Utah College of Medicine, Radiobiology Laboratory, Salt Lake City, Utah.

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TABLE I. Average Values for Radiopotassium Concentration in Tissues and Organs of Rats After Acute Whole Body Irradiation, Expressed as % of Administered Dose per g.

Organ		Days after irradiation or sham irradiation			Significance*		
		1	2	3	Control	Treated	Difference
Blood plasma	C†	.03	.03	.03	No	No	No
	I	.04	.03	.04			
Lymph nodes	C	.74	.68	.60	"	Yes	Yes
	I	.28	.45	.37			
Spleen	C	.74	.69	.71	Yes	"	"
	I	.67	.58	.64			
Stomach	C	.48	.37	.38	"	"	"
	I	.45	.30	.32			
Small intestine	C	.60	.44	.40	"	"	"
	I	.50	.26	.26			
Large intestine	C	.51	.34	.32	"	"	"
	I	.50	.26	.26			
Gonads	C	.56	.54	.62	"	"	No
	I	.57	.50	.57			
Lung	C	.45	.40	.40	"	"	"
	I	.44	.33	.34			
Liver	C	.57	.51	.55	"	"	"
	I	.56	.51	.61			
Kidney	C	.44	.43	.45	"	"	"
	I	.45	.39	.47			
Skin	C	.22	.24	.25	"	"	"
	I	.23	.21	.23			
Brain	C	.43	.42	.47	"	"	"
	I	.45	.40	.41			
Heart	C	.51	.50	.50	"	"	"
	I	.54	.45	.47			
Skeleton	C	.35	.32	.28	"	"	Yes
	I	.33	.21	.22			
Muscle	C	.74	.63	.76	"	"	No
	I	.75	.63	.72			
Red cells	C	.51	.61	.67	No	No	"
	I	.59	.56	.63			
Thymus	C	.79	.89	.47	"	"	Yes
	I	.23	.33	.33			

* Analysis of variance was performed upon data for each organ tabulated to determine whether the day to day changes in control or treated groups were significant. In addition the significance of the difference between the control and irradiated groups was checked. The results are given in the appropriate columns. A "yes" in the "control" or "treated" column, for example, indicates a significant change (P = .05) in concentration of K during experiment. A "yes" in the column headed "difference" indicates a similarly significant change in concentration of K in irradiated animals as compared to controls.

† C = control; I = irradiated.

X-ray machine for a time period similar to that for the exposed animals and in the same containers. The experimental and control animals were sacrificed at 24, 48 and 72 hours after irradiation and sham irradiation.

The rats were housed in standard metabolic cages. Urine and feces were separated and their volumes measured. At the time of sacrifice, the animals were anesthetized with ether, and heart blood was withdrawn into a heparinized syringe. The animals were then sacrificed by means of additional ether and the various tissues cleanly removed. The contents of the gastrointestinal tract were removed and the tissues were rinsed carefully in Tyrode's solution after which they were blotted dry on filter paper. All samples were weighed wet, assayed for radioactivity with a crystal counter and calculated as per cent of the administered dose per 24-hour specimen of urine or feces, or per gram of tissue.

Results. Radiopotassium concentrations per

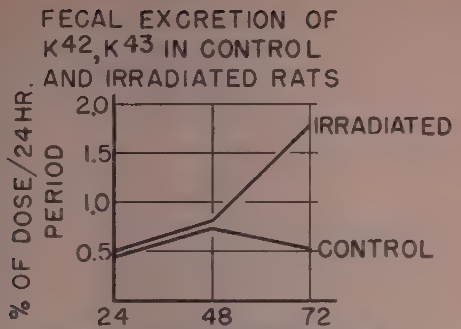

FIG. 1.

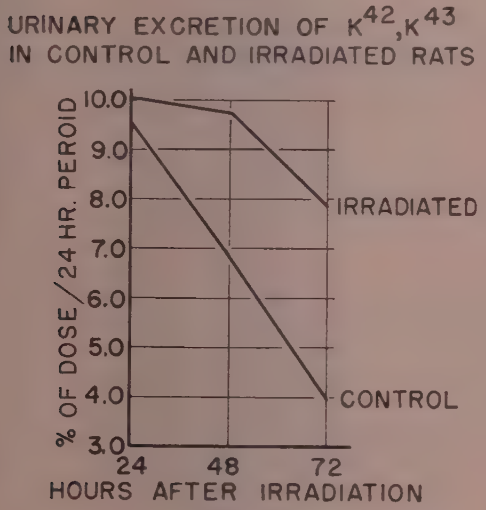

FIG. 2.

gram of tissue for a number of organs and tissues and the results of an analysis for variance are listed in Table I. The lymph nodes, thymus, spleen, stomach, small intestine, large intestine and bone exhibited a significant difference between control and heavily irradiated groups, as manifested by a reduction in the concentration of radiopotassium in the heavily irradiated group. The gonads were the only "sensitive" organ which did not follow this pattern, perhaps due to the comparatively small amount of "sensitive" germinal epithelium present. Fecal excretion of radiopotassium rose sharply with the onset of diarrhea at 72 hours post-irradiation (Fig. 1); urinary potassium levels were increased in the irradiated animals throughout the experiment (Fig. 2).

Discussion. Interpretation of our findings cannot include partition of potassium between extracellular and intracellular compartments since the data are calculated on a wet weight basis. Further studies have been initiated which it is hoped will furnish information necessary for more precise considerations of electrolyte partition.

In the subsequent paper in this series we report our results in generally similar experiments with radiosodium, and since these are closely related, some of the discussion is deferred to that paper. Several of our findings, however, deserve mention here.

On the basis of histopathological evidence, bone is reported to be a radiation-resistant tissue. Accordingly, we were impressed by the loss of potassium from bone in the irradiated animals. To determine whether this potassium was lost from bone, bone marrow or both, Dr. Virginia Davenport kindly carried out potassium determinations (flame photometer) on whole femur and on tibia with the marrow removed. The results (Table II) indicate that while there was a substantial loss from bone marrow—a sensitive tissue—there was the same degree of loss from bone with marrow removed. We assume that this may indicate a movement of potassium out of the bone lattice or a loss of potassium from osteoblasts, which are considered to be the most sensitive of the bone cells. If the latter explanation is correct, our data would suggest that osteoblasts are as sensitive to irradiation injury as are the cells of the hematopoietic system.

Dehydration, diarrhea, total anorexia and shock accompany whole-body irradiation injury in humans, and each of these manifestations has been reported to be associated with a depletion of intracellular potassium, particularly in muscle(5,6). In view of the severity of these manifestations in irradiation injury, we were impressed by our inability to detect more significant changes in potassium concentration in muscle during the 72 hours of the experiment.

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TABLE II. Total Potassium Content of Whole Bone and of Bone With Marrow Removed in Control and Irradiated Rats.*

Bone	Dry bone, m.Eq/kg		Fresh bone, m.Eq/kg	
	Control	Irradiated	Control	Irradiated
Whole femur	57.9	37.5	34.2	22.5
Tibia (marrow removed)	20.2	13	11.8	7.8

* As determined by flame photometry.

Urinary determinations, when compared with the control data, suggest that the potassium moving out of a number of tissues appeared rather promptly in the urine, and that the kidney does not conserve potassium in situations of developing intracellular deficit. Thus, total anorexia, depletion of potassium in a number of tissues and increased loss in stools and in the urine, all contribute to the fact that these animals were in absolute potassium deficiency.

Summary. Rats show a substantial loss of potassium from a majority of radiosensitive tissues beginning within 24 to 48 hours after irradiation. There is a comparable loss of radiopotassium from bone. Radiopotassium excretion is increased in the urine of irradiated rats, and there is a rise in fecal levels with the onset of diarrhea. It is evident that rats that have been irradiated at over an LD50 level develop an absolute potassium deficiency.

The authors wish to acknowledge the interest shown by Doctor Joseph G. Hamilton in this work.

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Distribution and Excretion of Electrolytes after Acute Whole-Body Irradiation Injury. II. Studies with Radiosodium.* (19170)

JOHN Z. BOWERS† AND KENNETH G. SCOTT. (Introduced by Louis S. Goodman.)

From the Crocker Radiation Laboratory and the Division of Radiology, University of California School of Medicine, Berkeley and San Francisco, Calif.

Several investigators have reported alterations in the sodium metabolism after acute whole-body irradiation injury. In rats, Painter(1) found an expansion of sodium space in the total animal and in the gastrointestinal tract five days after an LD50 exposure. Bennett *et al.*(2) noted a retention of radiosodium in rats with as much as 2% of the total dose accumulating in the gastrointestinal tract and smaller amounts in the kidney and spleen after an LD50 exposure. In the experiments of Bennett *et al.*, serum sodium levels rose by 5-8 mEq., and Kohn(3) has reported a plasma sodium rise of 3 mEq. on the 4th day after exposure just below an LD50. Soberman *et al.*(4) were unable to detect any significant alterations in plasma sodium in the dog after an LD50 exposure. Other investigators have reported a rise(5) or a fall(6) in blood sodium levels after irradiation injury.

This report concerns the pattern of urinary and fecal excretion of sodium in rats after acute whole body irradiation with X-rays and the accompanying alterations in sodium concentration in a number of tissues and organs.

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† University of Utah College of Medicine, Radiobiology Laboratory, Salt Lake City, Utah.

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2. Bennett, L. R., Bennett, V. C., and Howland, J. W., *Fed. Proc.*, 1949, v8, 350.

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Methods. Radioactive sodium (Na^{22}), supplied by Dr. Joseph Hamilton, was used as a tracer in doses of 2 microcuries injected subcutaneously 24 hours before irradiation. When required, urine and feces were separated in an animal holder modified after one suggested to us by Prof. W. O. Reinhardt. This device permits accurate separation of urine and feces despite diarrhea, and the only losses sustained are from the drying of liquid stools on the holder. Adult male rats of the Slonaker strain weighing approximately 250 g were used throughout.

In Exp. 1, 6 rats were irradiated and 6 held as controls; urine and feces were separated as described above. After 15 days the animals were sacrificed. In Exp. 2, the rats were kept in conventional cages and serially sacrificed immediately after irradiation (9 rats) and 24 hours (8 rats), 48 hours (9 rats), 72 hours (8 rats), 96 hours (8 rats), 120 hours (5 rats), and 9 days (5 rats) thereafter. At each period those rats were sacrificed which appeared most ill. Three rats were retained as controls and sacrificed on the 15th day. Irradiation dose in Exp. 1 was 710 r at 215 k.v., 15 m.a. at a rate of 77.4 r per minute with 1.2 mm Al and 0.5 mm Cu filters. In Exp. 2, the animals received 880 r, with similar factors and filters. The LD50 (30 das) for these animals was approximately 800 r.

After sacrifice, all tissues and organs were weighed wet and then dried at 104°C to a constant weight. Urine and feces were also dried at 104°C and all specimens were subsequently ashed in a muffle furnace. Ashed specimens were assayed for radioactivity by conventional Geiger-Müller counting procedures and the results calculated as per cent of administered dose per 24-hour specimen of urine or feces or per gram of wet tissue.

Results. In Exp. 1, diarrhea appeared in the irradiated animals on the 3rd day and persisted until the 7th day; tarry stools were noted on the 4th and 5th days. With the appearance of diarrhea, the concentration of radiosodium per gram of feces rose rapidly while the concentration per ml of urine fell, the two following each other in a complementary homeostatic manner (Fig. 1). As diarrhea subsided the urinary radiosodium concentrations rose to levels which were above those for the control animals.

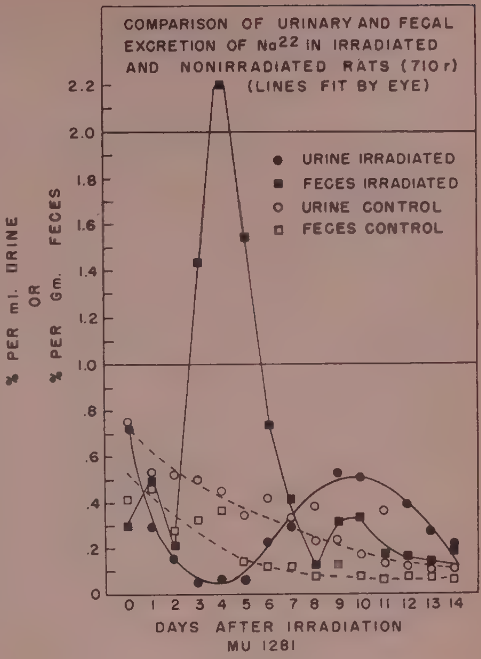

FIG. 1.

In Exp. 2, the time of appearance, character and duration of the diarrhea were similar to Exp. 1. Table I lists the control radiosodium concentrations and subsequent post-irradiation values expressed as per cent of control concentrations for a number of organs and tissues and for blood plasma. The lymph nodes showed an initial increase in concentration, then a transient decrease followed by a striking increase which reached a maximum value of 804% on the fifth day. In the gastrointestinal tract there was a decrease followed by an increase which was maximal on the fourth day, with a decrease to normal or sub-normal levels on the ninth day. The increase was most pronounced in the small intestine which histopathologically showed the greatest degree of radiosensitivity of the three segments of the gastrointestinal tract(7). The pattern of change in the spleen and testis was quite different from other "sensitive" organs.

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7. Warren, S., *Physiol. Rev.*, 1944, v24, 225.

TABLE I. Na^{22} in Tissues and Organs of Rats Receiving Acute Whole-Body Irradiation With 880 r.

		Avg values for Na^{22} conc., expressed as % of control values					
	Avg control values for Na^{22} conc. expressed as % administered dose/g tissue	Days after irradiation					
Tissue or organ		1	2	3	4	5	9
Blood plasma (%/ml)	1.19	110	103	113	106	104	122
Lymph node	.51	153	31.4	188	253	804	512
Stomach	.60	61.6	53.3	75	168	163	88
Small intestine	.43	102	62.8	198	228	198	93
Large intestine	.61	62.3	49.2	129	161	103	59
Spleen	.45	102	129	113	176	151	57.8
Testis	.62	103	77.4	46.8	103	74.2	102
Liver	.48	102	112	97.9	102	102	108
Kidney	.63	133	122	121	122	90.5	159
Skeleton	1.17	141	121	109	133	122	121
Muscle	.30	107	113	113	96.7	96.7	133
No. of rats	9	8	9	8	8	5	5

TABLE II. Total Sodium Content of Whole Bone and Bone Cortex of Control and Irradiated Rats.*

	Dry bone, mEq./kg		Fresh bone, mEq./kg	
Bone	Control	Irradiated	Control	Irradiated
Whole femur	221	245	132	147
Shaft tibia	235	236	139	142

* As determined by flame photometry.

There was a slight increase in radiosodium concentration in the spleen on the second day, a more pronounced increase on the fourth and fifth days and a decrease on the ninth day. The testis, in contrast, showed a decrease on the second, third and fifth days with a subsequent return to normal levels.

The liver did not exhibit any significant deviation from the control radiosodium concentration; this finding is in accord with the statement of Bloom(8) that the liver epithelium is highly radioresistant. The kidney which is considered to be "responsive but not sensitive"(7) showed an over-all slight increase in radiosodium concentration. There was also an over-all slight increase in skeletal radiosodium concentrations whereas muscle and plasma showed a slight rise on the ninth day. Sodium determinations (flame photometry) on whole bone and on bone shaft with marrow removed showed that the increase in radiosodium concentration found in whole bone at 48 hours after irradiation (Table II) could be accounted for by the increase in the highly radiosensitive bone marrow.

Table III lists the percentage water content of various organs before and on successive days after irradiation. All organs, with the exception of the lymph nodes which were not followed throughout, showed a rise on the ninth day. The spleen showed a decrease in water content on the first day and this was sustained until the terminal rise. Stomach, small intestine, large intestine, testis and liver showed an initial rise which was sustained or increased through the second day and which decreased toward normal levels on the third and fourth days. The kidney showed a decrease in water content on the fourth day.

Table IV shows the post-irradiation changes in dry weight, expressed as per cent of control values. Since there was considerable fluctuation in individual values, the per cent loss per day was also calculated from least squares fit of straight lines to percentages. Dry weight calculated as per cent loss per day by this method was most pronounced in lymph nodes, spleen, small intestine and testis. The spleen showed an initial striking increase in

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8. Bloom, W., *Histopathology of Irradiation from External and Internal Sources*, McGraw Hill, New York, 1948.

TABLE III. Percentage Water Content of Various Organs of Rats Before and After Acute Whole-Body Irradiation With 880 r.

Organ	Days 0	1	2	3	4	9
Lymph nodes	59	53.5	62.7	59.5	—	—
Spleen	81.9	70.5	73.7	71.7	70.2	78.3
Stomach	73.9	81.8	81.5	80.6	80.4	80.8
Small intestine	78.6	81.7	83.1	77.4	79.6	84.1
Large intestine	71.7	82.1	83.8	77.9	77.5	85.4
Testis	82.9	85.1	84.8	84.7	82.2	93.3
Liver	70	73	73.4	71	69.9	77.2
Kidney	77.5	75	77.2	75	71.8	80
No. of rats	9	8	9	8	8	5

The hypothesis that the table shows no effects of radiation with time or from organ to organ was tested and disproved (P = .001). The day to day and organ to organ differences in water content are significant at the 99.9% level (P = .001).

TABLE IV. Dry Weights of Various Organs of Rats Following Acute Whole-Body Irradiation With 880 r, Expressed as % of Control Values.*

Organ*	Days after irradiation 1	2	3	4	9	% loss per day†
Lymph node	96.2	50.9	35.8	32.1	—	19.6
Spleen	326	264	224	243	157	18.1
Stomach	78.3	67.7	74.6	85.4	60	3.11
Small intestine	83.5	64.8	67.8	72	65.8	7.80
Large intestine	72.8	—	65.3	56.6	45.2	5.16
Testis	93.5	87.6	81.2	96.2	32.2	7.25
Liver	98	90.2	88.9	73	68.4	3.76
Kidney	105	93.8	90.3	103	69.7	3.92
No. of rats	8	9	8	8	5	

* Percentages are expressed in terms of dry wt of organs of control group treated exactly as other groups but sacrificed immediately after irradiation. Figures are corrected for varying initial wt of rats, although this correction amounted to less than 5% for any one group.

† % loss per day was calculated from least squares fit of straight lines to percentages.

The hypothesis that the table shows no effects of radiation with time or from organ to organ was tested and disproved (P = .001).

dry weight followed by a persistent downward trend. The gastrointestinal tract showed a pronounced decrease in dry weight on the first two days whereas in the testis and kidney, there was a striking decrease on the ninth day only. The dry weight of the liver showed a steady downward trend.

Discussion. The discussion of our findings is obviously limited by the fact that concentrations are reported on a wet weight basis, as in the preceding paper. Experiments are in progress which should supply the information required for considerations of extracellular and intracellular concentrations.

Ionizing radiations primarily injure cells(7) and since potassium is the predominant intracellular cation and sodium the predominant extracellular cation, it is interesting to attempt to relate our findings with these electrolytes. In radiosensitive tissues, histopathological examination reveals extensive cellular destruction soon after exposure to irradiation levels such as were used in these experiments (7) and the loss of potassium from lymph nodes, thymus, gastrointestinal tract and spleen is undoubtedly related to the rapidity and degree of loss of function and structure in the sensitive cells in these organs.

The relation of the changes in radiosodium concentration to existing information on irradiation injury is more difficult, especially since they do not coincide with changes in percentage water content. It has been shown that sodium may move into cells at an increased rate in situations associated with a loss of intracellular potassium(9) and prob-

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9. Darrow, D. C., and Pratt, E. L., *J.A.M.A.*, 1950, v143, 365.

ably some of the striking increases in sodium concentration noted in several radiosensitive tissues are, in part, due to penetration into cells that have been injured by irradiation and from which there has been extensive loss of potassium.

The sharp decrease in radiosodium concentration which preceded the rise in lymph node and gastrointestinal tract is puzzling. However, it may be associated with the breakdown of large protein moieties into fragments with considerable osmotic activity. As these osmotically active fragments move out of injured tissues they may carry with them considerable amounts of sodium. That other factors are in operation is indicated by the lack of a decrease in the case of the spleen and the fact that there were no significant changes in radiopotassium concentration in the gonads when radiosodium levels fell sharply.

It is apparent that there is no correlation between the changes in percentage water content and in the dry weight of organs after irradiation. The early loss of dry weight on lymph nodes and gastrointestinal tract (Table IV) emphasizes the rapid decrease in tissue substance from specific radiation injury in these organs, while the later decrease in testis, liver and kidney probably reflects in part loss of tissue substance from the total anorexia that accompanies whole-body irradiation injury. The striking increase in dry weight of the spleen is puzzling. It may represent a pooling of formed elements of the blood in this organ and an accumulation of debris.

Edema has been reported as a prominent finding in cells injured by irradiation(7); the gastrointestinal tract and the testis showed an increase in water content at times when histopathological changes are reported to be pronounced. However, this increase in water content occurred when radiosodium and radiopotassium concentrations were decreasing, indicating that the increase was not the consequence of the movement of electrolyte into cells.

The increase in percentage water content found in all organs on the ninth day coincides with the time when diarrhea had subsided in the surviving animals and when they had resumed the intake of food and water.

The urinary excretory curves on Na^{22} (Fig. 1) demonstrate the ability of the kidney to defend sodium in the face of excessive loss in a sodium-rich diarrhea. In addition, the animals were totally anorexic at this time. The rebound increase in renal sodium excretion noted between the sixth and fourteenth days parallels the gradual resumption of food and water intake.

Summary. Radiosensitive tissues show a variety of alterations in radiosodium concentration after total body irradiation above an LD50. In several tissues there is a decrease followed by a sharp increase; the decrease occurs at the same time as a decrease in radiopotassium concentration. An increase in percentage water content may occur when there is a fall in radiosodium concentration. Percent loss of dry weight per day is in general most pronounced in radiosensitive tissues. Urinary excretion of radiosodium after total body irradiation at levels approximating an LD50 shows a complementary homeostatic decrease in response to the excessive loss of radiosodium through the gastrointestinal tract.

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Solubility of Bovine Fibrinogen in Ethanol-Water Mixtures.* (18171)

Peter R. Morrison, Sidney Shulman, and William F. Blatt.
From the Departments of Physiology, Chemistry and Zoology, University of Wisconsin, Madison.

The solubility of human fibrinogen in ethanol-water mixtures has been studied and on this basis there was developed a method for its purification from Fraction I, the crude concentrate formed by ethanol fractionation (1). The application of this procedure to the purification of bovine Fraction I has not been successful(2). This suggests that either the bovine fibrinogen or its impurities had different solubilities. It has also been observed that some of the properties of human and bovine fibrinogen differ slightly. For example, the bovine clotting time at pH 6.3 and ionic strength 0.45 is about twice as long and the opacity of the clot formed twice as great as the human. Further, a higher pH or ionic strength is required to cause the transition from production of coarse clots to production of fine clots(2-4). Rigidity measurements also reveal differences. At pH 7 and fibrin concentration 10 g/l, the rigidity of the human clot is about 6 times as great as that of the bovine; however, the rate of rigidity increase with increasing fibrinogen concentration is greater for the bovine material(6).

In view of these observations, it appeared worthwhile to study the solubility properties of bovine fibrinogen to establish differences and attempt to develop a satisfactory procedure for further purification.

Materials and methods. Armour bovine Fraction I was used as a source of fibrinogen. This material (lots C-107 and C-112) contained about 27% clottable protein. It was dialyzed at a fibrinogen concentration of about 20 g per liter against a buffer of 0.30 ionic strength (0.25 ionic strength sodium chloride plus 0.05 ionic strength sodium acetate or phosphate) for 20 hours with 2 changes of dialysis bath. Portions of this stock solution were then diluted with equal volume of (cold) diluent containing this same buffer composition and variable amounts of ethanol. After 3 hours of equilibration at 0°C the tubes were centrifuged and the supernatants decanted. The precipitates were redissolved at pH 6.3 and aliquots of these solutions and of the supernatants were clotted with thrombin in order to assay the fibrinogen(7). The supernatants and the redissolved precipitates were also analyzed for total protein, after an appropriate dilution, by measuring the light absorption at 2780 Å in the Beckman spectrophotometer. A linear relation between optical density (OD) and concentration (c) was obtained over the range, 0.05-1.25 g/l, such that OD = 1.476 c, as referred to Kjeldahl nitrogen determinations using a factor of 6.00(7).

Results and discussion. A representative series is shown in Fig. 1, where values for fibrinogen and total protein in the precipitate and supernatant phase as well as the sum of these 2 are plotted against ethanol concentration. Sum values remain satisfactorily constant for the various ethanol concentrations.

The results of the several series are summarized in Fig. 2, where the solubility of fibrinogen is shown in a 3-dimensional surface as a function of pH and ethanol concentration. Qualitatively this surface is similar to that found for human fibrinogen(1) in that it shows greater solubility at a high pH and low

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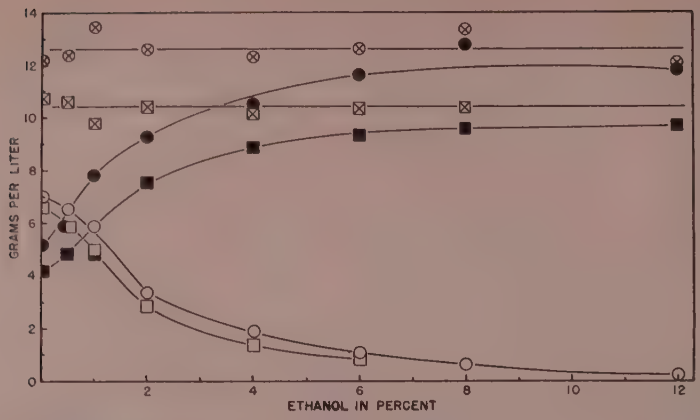

FIG. 1. The solubility of fibrinogen and total protein from Fraction I as a function of ethanol concentration. Temperature, 0°C; ionic strength, .30, phosphate (.05) and pH, 6.9. Squares, fibrinogen; circles, total protein; open symbols, precipitate; closed symbols, supernatant; half-closed symbols, sum of both phases.

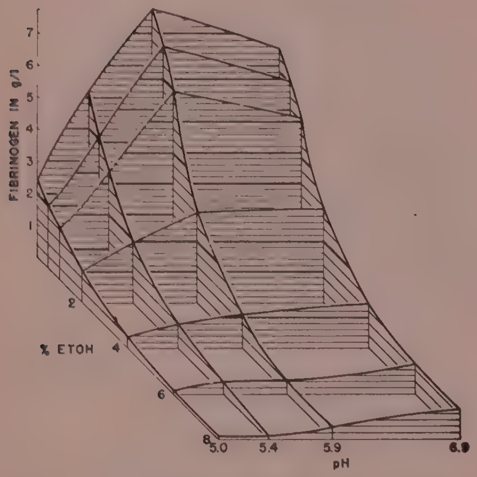

FIG. 2. The solubility of fibrinogen as a function of pH and ethanol concentration. Temperature, 0°; ionic strength, .30.

ethanol concentration. Quantitatively there are distinct differences in that the bovine fibrinogen shows a much smaller effect of pH with distinctly lower solubilities at pH 7 and higher solubilities at pH 5. The effectiveness of ethanol in reducing the solubility appears quite comparable in the two cases. This solubility behavior is in accord with the effect of pH on other properties of the clots formed from these fibrinogens. Thus in human fibrin clots there is a sharp change-over (at 0.15 ionic strength) from coarse to fine structured clots as the pH goes from pH 6 to pH 7. This correlates with a substantial shift in solubility in the human protein between these two pH values. On the other hand in the bovine substantially higher pH values must be reached before this transition takes place, and this appears in accord with the fact that little change in solubility was observed between pH 6 and pH 7 in the bovine fibrinogen.

Fractionation procedure. On the basis of the solubility results, a simple fractionation procedure was devised which gave a product with 90% clottability, as follows: Fraction I was dissolved in water to give about 3% protein. After cooling to 0°, an equal volume of cold 4% ethanol was added, containing 0.3 M sodium chloride and enough acetic acid to give a pH of 5.5 and a fibrinogen concentration of 1%. The precipitate was dissolved in 0.15 M disodium phosphate at 25° to give a pH of 7.6 and a protein concentration of about 2%. This was again cooled to 0° and an equal volume of cold 12% ethanol was added. The resulting precipitate was dissolved in the desired buffer solution at 25° to give the final solution. Yields of 30 to 35% were obtained. This procedure is similar to that developed for the human protein, but the middle step of removing a cold-insoluble fraction is omitted. Such a precipitate did not appear on simply cooling the solution of the first precipitate; this is in contrast to the behavior of the human Fraction I.

Summary. (1) The solubility of bovine fibrinogen in ethanol-water mixtures has been studied. The behavior was qualitatively similar to that of the human protein, but the influence of pH was much smaller. (2) A procedure for refractionating bovine Fraction I to give a 90% clottable product is described.

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Quantitative Studies on Prophylactic Effectiveness of Western Equine Encephalitis Antiserum in Mice.* (19172)

ALBERT B. SABIN.

From the Children's Hospital Research Foundation, University of Cincinnati College of Medicine.

The irregular, infrequent and unpredictable occurrence in the U. S. of sizeable epidemics of encephalitis due to the arthropod-borne viruses poses a difficult problem with regard to the various control measures which might be employed in the face of an epidemic. It has been demonstrated that the administration of suitable amounts of antiviral serum prior to infection, and under special conditions also at certain intervals after infection, can prevent development of the experimental disease (1-7). Previous studies were either of a qualitative nature in which large amounts of serum of unassayed potency were shown to have a protective effect, or, when sera of known potency were used in varying amounts, they were tested in animals infected by the intracerebral route. The purpose of the present communication is to present the results of certain pertinent quantitative studies which were carried out in 1942 and 1943 in relation to vaccination of human beings with western equine encephalitis chick embryo vaccine. The study was originally designed to indicate the significance of various levels of neutralizing antibody against western equine encephalitis virus as regards resistance to infection by a peripheral, extraneural route. The data, however, also throw light on the minimal amounts of passively introduced antibody which are required to protect against varying amounts of virus introduced by a peripheral, extraneural route, and thus provide a basis for estimating the amounts of antibody which may be required in humans.

Experimental procedure, methods and materials. The strain of WEE virus used was originally derived from California and since 1934 had been propagated in mice by Dr. P. K. Olitsky and his associates at the Rockefeller Institute and subsequently in this laboratory. The virus consisted of infected mouse brains, stored in dry ice as 10% centri-

TABLE I. Antibody Content of WEE Rabbit Antiserum Used in Prophylactic Tests.

Dilution of serum	Neutralization index* for .015 ml: Intra-abdominal test in 2-wk-old mice	Neutralization index* for .015 ml: Intracerebral test in 4-wk-old mice
Undiluted	5000000 + ?	1600
1:10	2000000	200
1:100	1000000 + ?	80
1:1000	6000	50
1:10000	13	6
1:100000	3	—
1:1000000	1	—

* Neutralization index = $\dfrac{\text{Titer of virus in mixtures with normal serum}}{\text{Titer of virus in mixtures with antiserum}}$.

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* This study was aided by the Commission on Virus and Rickettsial Diseases of the Armed Forces Epidemiological Board.

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TABLE II. Intracerebral Neutralization Tests on Sera of Mice Inoculated with WEE Rabbit Antiserum.

Route of inoculation	Amt of antiserum inj.	Avg wt of mice inoc., g	Time between inj. of antiserum and withdrawal of mouse serum, hr	Original dilution of mouse serum tested	Neutralization index (.015 ml in test)[*]
Fresh normal mouse serum control			—	U[†]	1
Intra-abdominal	.5 ml undilut.	18	4	U	320
			24	U	1300
			48	U	5000
	.15 ml of 1:10	11	4	U	1000
			24	U	500
			48	U	630
	.03 " " 1:10	10	4	U	500
			24	U	200
			48	U	320
Intravenous	.15 " " 1:10	7.2	4	U	500
				1:10	400
			72	U	800
	.15 " " 1:100	7.2	4	U	500
				1:10	10
	.15 " " 1:1000	7.3	4	U	63
				1:10	1
	.15 " " 1:33	19.5	4	U	160
				1:10	16

[*] This means that the .03 ml of serum-virus mixture inoculated contained .015 ml of serum.
[†] U = undiluted.

fuged suspensions in heated, undiluted rabbit serum. The intracerebral titer in mice of the freshly prepared suspension, diluted in 10% heated rabbit serum saline, was as a rule 10^{-9} per 0.03 ml. In intracerebral neutralization tests, following incubation for 2 hours at 37°C in the presence of equal parts of heated undiluted rabbit serum, the titers varied from $10^{-8.2}$ to $10^{-9.0}$. The WEE antiserum was prepared in 3 rabbits, each weighing 2.4 to 3.0 kilos, by a series of 5 inoculations of frozen or fresh, 10% mouse brain suspension according to the following schedule: 0.3 ml subcutaneously on 8/29/42; 0.3 ml intravenously on 9/8/42 and 9/10/42; 3 ml subcutaneously on 9/12/42 and 6 ml subcutaneously on 9/14/42; the rabbits were bled on 9/24/42 and the serum was pooled. Both the intracerebral and intra-abdominal neutralization tests were used to assay the potency of this antiserum by testing mixtures consisting of 10-fold dilutions of the serum and various 10-fold dilutions of the virus incubated for 2 hours at 37°C prior to inoculation (Table I). The dilutions of antiserum for these tests were made in heated undiluted rabbit serum to provide optimum protection for the diluted virus during the period of incubation at 37°C. Varying amounts of this antiserum were also injected intra-abdominally or intravenously in mice of known weight, which were subsequently bled at intervals, indicated in Table II, to determine the minimal amount of serum which had to be injected to yield an intracerebral neutralization index of approximately 50 in the undiluted mouse serum (Table II).[†] The final evaluation of the limits of the prophylactic effectiveness of the antiserum was carried out in various groups of approximately 2-week-old mice (weighing about 7 g) which were first inoculated intravenously with varying amounts of normal or WEE rabbit serum diluted in saline, and 4 hours later received an intra-abdominal injection of 0.3 ml of varying dilutions of WEE virus (Table III).

Results. The assay of the antiserum, shown in Table I, indicates that 0.015 ml of the

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[†] The rabbit antiserum was itself lethal for mice within 10 to 20 minutes when given intravenously in doses of 0.15 ml of undiluted or 1:2 diluted serum, while 0.15 ml of the 1:10 or greater dilutions was harmless. By the intra-abdominal route 0.5 ml of the undiluted antiserum was harmless. The mouse-tissue antibodies contained in the antiserum may have been responsible for the toxic effect of the larger doses.

TABLE III. Limits of Prophylactic Effectiveness of WEE Rabbit Antiserum in Mice Serum Intravenously 4 Hr Before Virus. Virus Intra-abdominally—.3 ml.

		Serum administered				
Date of test	Type of serum	Dilution of serum inj. in vol. of .15 ml	Avg wt of mice, g	Dose per kilo, ml	Intra-abdominal titer of virus in indicated groups of mice*	Protective index of antiserum log§
10-17-42	None—control	—	7.3	—	7.5†	—
10-23-42	Normal rabbit	1:10	7.2	2	6.4†	—
	WEE	1:10	7.2		1.4	5
1-21-43	Normal	1:100	7.2	.2	6.4‡	—
	WEE	1:100	7.2		2.2	4.2
2-8-43	Normal	1:1000	7.3	.02	5.8‡	—
	WEE	1:1000	7.3		3.2	2.6
	Normal	1:33	19.5	.23	1.4	—
	WEE	1:33	19.5		.8	.6

* Groups of 5 mice were tested at each serial 10-fold dilution of virus in the range of 10^{-1} to 10^{-9}, and the titer represents the reciprocal of the log of 50% endpoint for the .3 ml dose.

† Same lot of virus used on 10-17-42 and 10-23-42 with intracerebral titer of 10^{-9}/.03 ml.

‡ Same lot of virus used on 1-21-43 and 2-8-43 with intracerebral titer of $10^{-8.5}$/.03 ml.

$$\S\ \text{Protective index} = \frac{\text{titer of virus in mice inoculated with normal serum}}{\text{titer of virus in mice inoculated with antiserum}}.$$

1:1,000 dilution was the limiting effective amount in both the intra-abdominal and intracerebral neutralization tests. It should be noted that this antiserum was relatively ineffective against larger amounts of virus by the intracerebral route. Thus, when the intracerebral 50% serum dilution endpoint for this antiserum was calculated from the same data by the method of Reed and Muench, it was 1:1,800 against 40 LD_{50} of virus and only 1:3 against 400 LD_{50} of virus. It is evident, however, that, when small doses of virus are used, the less cumbersome intracerebral test can be used for comparative assay of WEE antisera. While the intra-abdominal test yielded higher neutralization indexes at any given dilution of serum, it did not yield a higher serum-dilution protective endpoint.

The data presented in Table II indicate a good correlation between the neutralization index obtained in the regular intracerebral neutralization test and that obtained after the antiserum was diluted in the bodies of mice of known weight. Thus, 0.15 ml of the 1:1,000 dilution of antiserum given intravenously to mice weighing 7.3 g (0.02 ml/kg), after an interval of 4 hours, yielded a mouse serum with an intracerebral neutralization index of 63 in the undiluted state and no neutralization in the 1:10 dilution. Since 0.015 ml of the 1:1,000 dilution of the antiserum yielded a similar result in the original neutralization test (Table I), it would appear that the 0.15 ml inoculum underwent a dilution not greater than 10-fold in the bodies of 7.3 g mice. It is thus evident that the antibody neither gains nor loses any significant activity within a few hours after intravenous injection.

The data summarized in Table III show that: (a) the smallest amount of antiserum used (0.02 ml/kg) yielded a protective index of 400, *i.e.*, protected the mice against 400 times more virus than did a similar injection of normal rabbit serum; the actual amount of virus resisted by 50% of the mice was approximately 2 million (mouse) intracerebral lethal doses; (b) roughly proportionately larger amounts of antiserum were required to protect against larger amounts of virus; thus, 0.02 ml, 0.2 ml, and 2 ml of antiserum per kilo yielded protective indexes of 400, 16,000 and 100,000 respectively; (c) when older mice were used which, because of their natural maturation resistance, required very large amounts of virus for the production of encephalitis by the intra-abdominal route, much larger amounts of antiserum were needed to protect against the minimal intra-abdominal encephalitogenic dose.

Discussion. The data, here presented, emphasize some of the factors that need to be taken into consideration in any attempt to determine a suitable dose of antibody for the protection of human beings during an epidemic of arthropod-borne virus encephalitis. Thus, the dose of virus is important, but there is, as yet, no precise information on the number of mouse intracerebral infectious doses of virus that mosquitoes transmit, nor on the number of such doses that are required to produce encephalitis in the most susceptible human beings. If the minimal human encephalitogenic dose corresponds to that obtaining for 2-week-old mice, 0.02 ml per kilo of an antibody solution, possessing the potency of the serum used in these tests, might be protective at the moment of inoculation, while 100 times as much or more may be required if the human encephalitogenic dose of virus should be much larger. The ideal dose of antiserum would, of course, be one which would supply enough antibody to provide protection for the whole period of potential exposure against the largest amounts of virus that mosquitoes might be able to transmit. The minimum dose of antiserum that might be considered for practical trial in human beings should provide enough antibody to be just detectable in the individual's undiluted serum at the end of the period of potential exposure.

The concentration of antibody achieved in the rabbit serum used in the present tests is not necessarily maximal, although Olitsky, Schlesinger and Morgan(6) have pointed out that merely increasing the number of inoculations beyond a certain minimum does not yield a WEE antiviral serum of greater potency. Nevertheless, it is possible that by the use of adjuvants and properly spaced booster inoculations in horses, cattle, pigs and sheep antiviral sera of greater potency than that used in the present tests might be prepared. Furthermore, concentration of approximately 15 to 30 times might be achieved by precipitation of the antibody with the gamma globulin fraction of these sera. Further studies are, therefore, indicated to determine whether or not antibody solutions can be prepared which are at least 100 times more potent than the serum used in the present tests. Such concentrated preparations, in doses of 0.02 to 0.1 ml per kilo, should provide sufficient antibody to warrant more detailed studies in human beings.

Summary. (1) Hyperimmune, WEE antiserum, prepared in rabbits by a series of 5 fairly closely spaced inoculations, yielded a 50% serum dilution titer of 1:1,800 against 40 LD_{50} of virus in the routine intracerebral neutralization test. When the intra-abdominal neutralization test was used, the amount of virus neutralized by various dilutions of the serum was always greater, but the limiting effective dilution of the serum was about the same as in the intracerebral assay against 40 LD_{50} of virus. (2) After intravenous injection of this antiserum in mice, the mouse serum acquired a neutralizing activity which indicated that the antibody underwent a dilution roughly in proportion to the size of the animal. (3) Prophylactic effectiveness was tested by injecting intravenously various dilutions of antiserum into 2-week-old mice, and 4 hours later inoculating these mice intra-abdominally with varying dilutions of the virus. Passive transfer of just enough antibody to impart to the undiluted serum an intracerebral neutralization index of approximately 50, protected against 400-4,000 intra-abdominal encephalitogenic doses of virus; the actual amount of virus contained in this dose was 2 million (mouse) intracerebral LD_{50}. The amount of antibody required for passive protection varied with the size of the infecting dose, so that 0.02 ml, 0.2 ml and 2 ml of the antiserum per kilo of body weight yielded protective indexes of 400, 16,000 and 100,000 respectively. The significance of these data for the problem of the prophylactic use in human beings of concentrated antisera against the arthropod-borne encephalitis viruses is discussed.

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Interrelationships Observed Between Lansing Strain of Poliomyelitis Virus and Virus of Sporadic Bovine Encephalomyelitis.* (19173)

Te Wen Chang and Herbert A. Wenner.

From the Department of Pediatrics and the Hixon Memorial Laboratory, University of Kansas School of Medicine, Kansas City, Kansas

McNutt and Waller(1) described an acute infectious disease of cattle which is now referred to as sporadic bovine encephalitis. The disease was characterized by fever, loss of appetite, weight loss, and weakness of the hind limbs(2). As a rule, cattle less than 3 years of age were attacked. NcNutt(2) and Harshfield(3) have detected in brain tissues obtained from infected calves a filterable agent which was able to reproduce the disease. The causative agent has not been grown in artificial media, but it can be passaged in series in the yolk sac of developing chick embryos, and in the peritoneal cavity of guinea pigs. Although not completely defined, the agent appears to be a filterable virus.

Two strains of bovine encephalitis virus (BEV) have been studied in this laboratory in an effort to define a relationship between them and some other known neurotropic viruses affecting human beings. These 2 strains have been adapted successfully to Swiss mice, cotton rats, and *rhesus* (M. mulatto) monkeys Serologic tests have indicated a relationship between the virus of sporadic bovine encephalitis and the Lansing strain of poliomyelitis virus. The results of these experiments are described herein.

Materials and methods. Viruses. The "L" and "S" strains of sporadic BEV were received on November 18, 1950 from Dr. G. S. Harshfield.† Each of these strains was passaged in 7-day-old chick embryos on March 7, 1951. The yolk of moribund embryos was harvested 4 to 6 days later. Bacteria-free aliquots (2 ml) of each of the yolk pools were stored at —70°C. These materials were used in the experiments reported here. The Lansing strain of poliomyelitis virus was received from Dr. Charles Armstrong‡ on September 13, 1946, and represented the 332nd passage in mice. The virus used in the present study represented the 334th passage prepared as a pool in this laboratory on October 23, 1950.

Serums. Serums were obtained from *rhesus* monkeys before and following convalescence from infection with BEV. Immune serums were also obtained by the repeated inoculation of infected yolk emulsion and mouse brain extracts mixed with adjuvants(4). Antiserums were prepared (1948-1950) in *rhesus* monkeys with the Lansing strain of poliomyelitis virus,§ the WE strain of lymphocytic chorio-meningitis virus, Western equine encephalitis (WEE), and St. Louis encephalitis viruses. These antiserums of known titer were used also in neutralization tests. *Animal passage.* Each strain of BEV was passaged intracerebrally in Swiss mice, cotton rats, hamsters, guinea pigs, rabbits, and *rhesus* monkeys. In the event of apparent illness, or the appearance of neurological symptoms, animals were sacrificed for purposes of (a) histologic study, and (b) the passage of CNS emulsions into other animals. All animals were observed 30 days unless they were sacrificed earlier. *Neutralization tests.* In the

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* Aided by a Grant from the National Foundation for Infantile Paralysis, Inc.

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† We are indebted to Dr. G. S. Harshfield, South Dakota State College, Brookings, South Dakota, for his kindness in making available to us the "L" and "S" strains of sporadic boxine encephalitis virus.

‡ We are indebted to Dr. Charles Armstrong, National Institute of Health, Bethesda, Maryland for the Lansing strain of poliomyelitis virus.

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§ The Lansing antiserum, A-300, was prepared by Dr. Jonas Salk at the University of Pittsburgh, Pittsburgh, Pa.

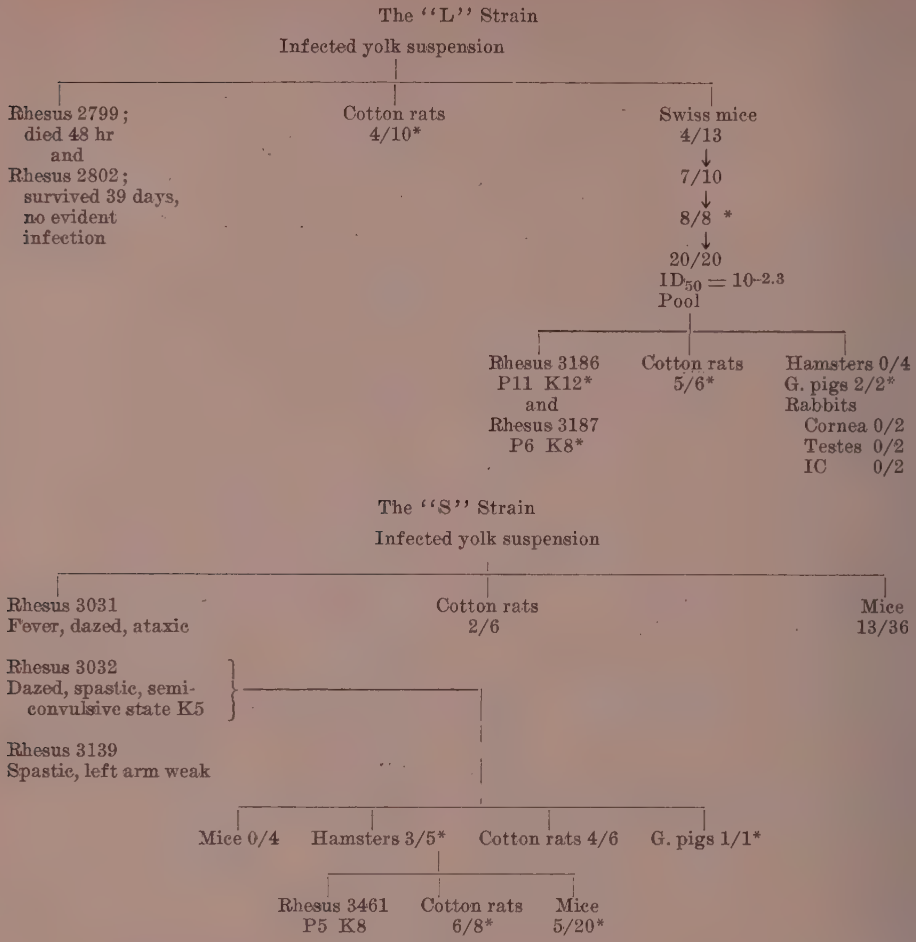

FIG. 1.

P5 = paralyzed, 5th day; K8 = killed, 8th day. * Clinical encephalomyelitis and positive histology.

serum-virus neutralization tests serial 4-fold dilutions of serum were tested against 50 or 100 ID_{50} of virus. The ID_{50} of the mouse-adapted "L" strain of BEV was $10^{-2.3}$. The ID_{50} of the Lansing strain of poliomyelitis virus was $10^{-3.9}$. Serum-virus mixtures were incubated 2 hours at 37°C. Each serum-virus mixture was inoculated into six 6-week-old Swiss mice. Appropriate controls were included. Mice were observed and scored over a period of 28 days.

Results. Adaptation of BEV to rodents and rhesus monkeys. The "L" and "S" strains of BEV caused encephalomyelitis in *rhesus* monkeys, cotton rats, and Swiss mice. Guinea pigs and hamsters experienced encephalomyelitis also after intracerebral inoculation with virus; however, infection occurred less regularly than in aforementioned animals. Domestic rabbits were not apparently affected following intracerebral or intratesticular inoculation, or by the application of virus to the scarified cornea. The "L" strain of BEV was adapted to mice, causing encephalomyelitis after cerebral inoculation. The infectivity titer was determined at the fourth passage in mice; the ID_{50} was $10^{-2.3}$. On passage in the brain of monkeys and cotton rats, the mouse-

TABLE I. Results of Neutralization Tests with BEV and Antiserums of Known Titers.

Antiserum	Homotypic serum titer	Dilution of serum 1:8	1:32	1:64	1:256	Serum protective index
WEE	1:8000	6/6*	6/6	6/6	6/6	<1:8
St. Louis	1:1000	5/6	5/6	6/6	6/6	<1:8
BEV	1:256	1/6	1/3	1/6	2/4	1:128
Lansing, A-300	>1:1024	1/6	1/6	1/6	0/6	>1:256
Normal monkey serum	<1:4	6/6	4/6	6/6	6/6	<1:8

* Numerator = No. dead or surviving; denominator = No. inoculated.
50 ID_{50} of ‘‘L’’ strain of BEV was used in the test.

TABLE II. The Neutralization of the Lansing Strain of Poliomyelitis Virus by BEV Immune Monkey Antiserums.

Rhesus monkey No.	Strain and antigen used for hyperimmunization	Dilution of serum 1:2	1:8	1:32	1:128	1:256	Serum protective index
2802	‘‘L’’ yolk	6/6	6/6	5/6	6/6	5/6	<1:2
2810	‘‘L’’ ’’	—*	5/6	5/6	6/6	6/6	<1:8
2944	‘‘L’’ mouse brain	0/6	1/5	1/6	0/3	2/6	1:256
2947	‘‘L’’ ’’ ’’	0/6	0/6	1/6	3/6	3/6	1:200
3064	‘‘S’’ yolk	6/6	5/6	6/6	6/6	6/6	<1:2
3065	‘‘S’’ ’’	5/6	6/6	4/5	5/6	4/6	<1:2
A-300	Lansing, CNS	—	0/6	1/6	—	—	>1:32
Normal Pool #2	None	—	6/6	5/6	—	—	<1:8

100 ID_{50} was used.
* Mice escaped and were killed.

adapted virus produced illness and subsequent appearance of flaccid paralysis. It also provoked illness in guinea pigs, but failed to affect four hamsters. Difficulty in re-establishing the strain in chick embryos was met with. As stated earlier, rabbits were not visibly affected by the mouse-adapted strain of BEV. The "S" strain of virus was adapted also to monkeys, hamsters, mice, and cotton rats. The monkey-adapted virus caused encephalomyelitis in hamsters, cotton rats, and guinea pigs. The hamster-adapted virus in turn caused paralysis in mice, cotton rats, and a *rhesus* monkey. The "S" strain was somewhat more difficult to adapt to mice than the "L" strain, but this was accomplished with hamster-adapted BEV. A diagrammatic sketch of some of the passages made in rodents and in monkeys appears in Fig. 1.

Histologic pathology. The CNS of mice, cotton rats, and monkeys showed meningoencephalomyelitis. There was cellular damage both of neurones and supporting tissue framework In monkeys the lesions in the spinal cord were indistinguishable from those seen in poliomyelitis.

Neutralization tests. Four neutralization tests were done. The "L" strain of BEV was used in the tests. *In ovo* and mouse neutralization tests gave corresponding results. Neutralization of BEV was not observed in tests made with WEE, St. Louis encephalitis, and lymphocytic choriomeningitis antiserums. The results obtained in one of the tests appear in Table I.

It is evident from the data in Table I that BEV was neutralized by the homologous antiserum, and in addition by antiserum obtained from monkeys immunized with the Lansing strain of poliomyelitis virus. In order to study this relationship, six monkeys were immunized with BEV yolk suspensions ("L" and "S" strains) and the mouse-adapted "L" strain of BEV. These antiserums were then tested in order to determine their capacities, respectively, to neutralize the mouse-adapted Lansing strain of poliomyelitis virus. The results of the tests appear in Table II.

Monkeys immunized with mouse-adapted BEV had a high titer of serum antibody (serum dilution, 1:200 or >) able to neutralize the Lansing strain of poliomyelitis virus. Monkeys immunized with egg-yolk suspension containing virus ($ID_{50} = 10^{-3.7}$) failed to develop neutralizing antibodies. In the latter monkeys, BEV antibodies were not found both *in ovo* and in mouse neutralization tests. The differences in the two antigens in regard to their capacities to form antibodies are being studied further.

Discussion. These observations on BEV are recorded because: (a) the virus has been adapted to new mammalian hosts, and (b) there appears to be an immunologic relationship between it and the Lansing strain of poliomyelitis virus. It should not be inferred from these data that BEV is caused by poliomyelitis virus. Further studies, now in progress, should clarify the nature of the viral agent. There is evidence(*2,5*) that the infectious agent of BEV may be a member of the psittacine group of viruses, since elementary bodies have been observed(*2,6*) in the exudates of calves sick with the disease, and in infected tissues derived from animals inoculated with BEV.

These observations are of additional interest, because they would explain heretofore perplexing observations regarding the occurrence of neutralizing antibodies for the Lansing strain of poliomyelitis virus in the body fluids of cattle(7) and other domestic birds and animals.

Summary. Two strains of bovine encephalitis virus have been adapted to mice, cotton rats, and *rhesus* monkeys causing meningoencephalomyelitis. Evidence was presented to show that the Lansing strain of poliomyelitis virus and the bovine encephalitis virus are related serologically.||

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|| We have considered carefully the possibility of contamination of infected yolk suspension with the Lansing strain of poliomyelitis virus. Against this likelihood has been the fact that BEV has been passaged many times in chick embryonic tissues, that each of the strains studied caused encephalitis in hamsters and guinea pigs, and finally, that the egg-adapted strain was neutralized by Lansing serum as well as the mouse-adapted.

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Use of Trichloracetic Acid in Purification of Lipids.* (19174)

R. M. JOHNSON AND PAUL H. DUTCH. (Introduced by A. H. Smith.)

From the Detroit Institute of Cancer Research, Detroit, Mich.

Several methods have been developed for the removal of impurities, chiefly nitrogenous, from lipid extracts of animal tissues(1-3). That of McKibbin and Taylor(3) has been found in this laboratory to be the most satisfactory from the standpoint of general application and completeness of purification with minimal loss of lipid. The number of operations required in this method, however, reduces its desirability for use with micro quantities, and in work involving radioactive tracers. Attempts to find a suitable procedure indicated that removal of the acid-soluble material from a tissue with 10% trichloracetic acid (TCA) prior to extraction with lipid solvents may facilitate purification of the lipid extract. Swanson and Artom(4) reported the use of TCA in a similar manner, but they noted that when tissues were ex-

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tracted with lipid solvents without previous use of TCA, higher values for the weight of lipid were obtained. It has been found that this discrepancy can be overcome by using 10% TCA containing 0.4 M $MgCl_2$.

The procedure that has been adopted, and which has been used in this laboratory for purifying lipids from samples of tissues weighing as little as 0.3 g, is as follows. The tissue is removed from the animal and immediately transferred to an all-glass homogenizer of the Potter-Elvehjem type. Cold 10% TCA containing 0.4 M $MgCl_2$ is added, about 5 ml being used for 0.5 g or less of tissue. The homogenizing tube is held in an ice bath, and the sample rapidly homogenized. It is centrifuged in the cold, and the residue is washed 3 times with cold 5% TCA containing 0.4 M $MgCl_2$, with centrifuging after each washing. The residue is then extracted in the usual manner with boiling alcohol, followed by 3 extractions with ethanol-ether (3:1). The extracts are pooled, and the solvent removed under nitrogen in a partial vacuum, keeping the temperature below 55°C. The near-dry residue is reextracted with boiling petroleum ether-chloroform (6:1), and the extract washed with water and dried.

Tissues treated in this manner have been digested with NaOH, followed by acidification and re-extraction with lipid solvents. The failure to recover additional lipid indicated that the original extraction was complete. This method has been compared with that of McKibbin and Taylor, and also with a method identical in all respects except that the acid-soluble material was not removed with TCA prior to extracting the lipids. In this study aliquot weights of the same tissue were used, and the lipids were compared on the basis of amount found (Bloor's oxidative procedure) (5), I_2 number, free fatty acid content, and N:P ratios. The present method and that of McKibbin and Taylor resulted in similar yields of total lipid (Table I). Lipids prepared by these two methods possessed N:P ratios that were similar, and considerably lower than those found in the unpurified samples. Furthermore, the ranges of the ratios were similar and much narrower in the

TABLE I. Effect of Purification on Lipids Obtained from Various Tissues. (No. in parentheses indicate ranges).

		Method A*			Method B*			Method C*		
Tissue	No. of exp.	Total lipid†	I_2 No.	N:P	Total lipid†	I_2 No.	N:P	Total lipid†	I_2 No.	N:P
Liver	5	4.3 (4.2-4.5)	105 (101-108.2)	.54 (.47-.61)	4.2 (4.1-4.3)	102 (89-117)	.53 (.45-.63)	4.5 (4.2-4.7)	106 (98-112)	.69 (.56-.85)
Lung	5	3.5 (3.1-5.4)	79 (76-82)	.58 (.56-.63)	3.4 (3 -5.2)	74 (68-83)	.58 (.49-.68)	3.2 (2.6-5.9)	82 (77-95)	.75 (.62-.97)
Brain	5	7.8 (6.2-8.3)	79 (71-86)	.71 (.60-.76)	7.7 (6.4-8.1)	86 (80.2-94.2)	.68 (.58-.74)	8.5 (7.4-8.7)	84 (80-90)	.85 (.67-1)
Tumor	2	2 (1.9-2.1)	92 (91.8-92.6)	.50 (.47-.53)	2.1 (1.9-2.3)	90 (86-94)	.52‡	2.4‡	105‡	1.15 (.97-1.33)

* Method A: Described herein. Method B: That of McKibbin and Taylor (3); the $CHCl_3$ extracts were washed 6 times. Method C: Identical to method A except that acid-soluble materials were not removed with 10% TCA.

† Total lipid expressed as % of wet weight of tissue.

‡ Single observations.

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case of the purified than in the unpurified samples. The difference is due mainly to decreased nitrogen content of the purified samples, as one might expect in view of the findings of others that the impurities present are largely those of nitrogenous nature(6-9). The lipids resulting from both purification processes may possess lowered I_2 numbers. The use of TCA did not result in measurable hydrolysis of the lipids, as indicated by a determination of free fatty acid content.

Summary. A method is described for the quantitative extraction and purification of small amounts of lipid with a minimum of manipulation. The acid-soluble material is removed from the tissue with cold 10% trichloracetic acid containing 0.4 M $MgCl_2$. The lipid is removed from the residue using ethanol-ether. This extract is taken to near-dryness, re-extracted with petroleum ether-chloroform, and the latter solution washed with water and dried. The lipid thus prepared has been found to compare favorably in several respects with lipid purified according to McKibbin and Taylor(3).

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